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Molecular mechanisms of cisplatin-induced neurotoxicity: formation and repair of specific DNA lesions in different cell types of nervous tissue

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Makhalova, J., Limmroth, V., Katsarava, Z., Liedert, B., Koeppen, S., Thomale, J. (2001): Platin-DNA-Addukte: Neue Marker der Cisplatin-Neurotoxizität. Akt. Neurol. 28(Suppl.2), 121.

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CONTENTS

1 INTRODUCTION

1.1 Drug-induced neuropathy

Peripheral neuropathy is a side effect of many drugs used in the treatment of a variety of disorders, including cancer, infections, epilepsy, connective tissue disorders and cardiac arrhythmias. The severity of this complication may range from some loss of sensory function and mild paraesthesias to neuropathic pain, severe ataxia and weakness leading to pronounced disability. The involvement of autonomic nerve fibers with orthostatic hypotension, impotence and incontinence may further reduce the quality of life (Hilkens & van den Bent, 1997).

Especially cancer patients, undergoing chemotherapy with platinum-derivatives, taxoides or vinca-alcaloides are often left with permanent evidence of peripheral nerve damage (Schattschneider et al, 2001; Screnci & McKeage, 1999). With regard to this neurotoxic effect, total dose is restricted, which may result in therapeutic failure.

Although most of anti-cancer drug induced neuropathies have been extensively described clinically over the past 20 years, little is known about the pathophysiological mechanisms. Elucidating of these mechanisms might enable oncologists in the future to counteract neuronal damage with new chemoprotective strategies.

1.2 Use and obstacles of platinum based chemotherapy

1.2.1. Indication

The platinum complex cisplatin (cis-diamminedichloroplatinum [II]), first described as an antineoplastic agent in 1965 by Rosenberg et al., is among the most frequently used chemotherapeutics, indicated against a broad range of solid tumors, which primarily were testicular, ovarian and bladder neoplasms (Cersosimo, 1989; Higby et al., 1974). Recently the spectrum of indications has been expanded including now endometrium-, cervix-, prostate-carcinomas; osteosarcomas as well as head and neck tumors (Petsko, 1995). Additionally, this substance has shown its efficacy against small cell and non-small cell lung cancer (Eberhardt et al., 1998; Havemann & Wolf, 1997) and is being used in the therapy of gastrocarcinomas as well (Kath et al., 2000; Konishi et al., 1998).

Cisplatin is administered intravenously in 3-5 courses spread over several weeks to months. Individual courses are usually 20-40 mg/m^2 (Windebank, 1996).

1.2.2 Unwanted side effects

The use of platinum compounds is associated with several systemic adverse events. Short term phenomena like nausea and vomiting are seen in nearly all patients treated with cisplatin (Hamers et al, 1991).

More threatening are the effects of platinum complexes, which appear at higher cumulative doses. In former times, the dose escalation of cisplatin therapy was limited in first instance by renal toxicity manifested as a marked reduction of glomerular as well as tubular function in 15-30% of patients (Cornelison & Reed, 1993).

Such functional impairment causes protein- and glucosuria, hyperuricaemia and hypercreatininaemia, serum electrolyte disturbances and is a serious risk factor of an acute kidney failure (Lau, 1999). Both renal and gastrointestinal side effects, however, have been reduced considerably in the clinic by several measures such as careful saline hydration (Alberts & Noel, 1995) as well as the use of antiemetics (Hamers et al, 1991; Screnci & McKeage, 1999). Thus, cisplatin-induced neurotoxicity represents now the predominant complication during cisplatin chemotherapy.

1.3 Neurotoxicity – the major dose limiting factor of cisplatin chemotherapy

About 20% of the patients are unable to complete a full course of cisplatin chemotherapy due to neurological damage (Cano et al., 1998; McDonald & Windebank, 2002). The spectrum of cisplatin-induced neurotoxicity includes peripheral sensory neuropathy (Alberts and Noel, 1995; Boogerd, 1995; Cersosimo, 1989; LoMonaco et al., 1992; Hamers et al., 1991; Hilkens & van den Bent, 1997; Meijer et al., 1999), ototoxicity (Alberts and Noel, 1995; Smoorenburg et al., 1999), and, rarely, autonomic neuropathy with orthostatic hypotension and gastric paresis, Lhermittes symptom (Cersosimo,1989), optic neuropathy (Cersosimo, 1989; Hilkens & van den Bent, 1997), focal encephalopathy, cortical blindness and seizures (Alberts and Noel, 1995; Cersosimo, 1989; Hilkens & van den Bent, 1997).

Of these, peripheral sensory polyneuropathy (PNP) is by far the most common dose limiting toxicity (Hadley et al., 1979; Ozols & Young, 1984; Roelofs et al., 1984; Thompson et al., 1984).

1.3.1 Clinical signs

PNP was first reported as a side effect following cisplatin administration by Kedar et al. in 1978. The actual literature data about the incidence of cisplatin-associated neuropathy are varying from 30 to 100% of treated patients, most likely due to the differences in patient populations, therapy protocols and evaluation methods used. The overall incidence of any grade of neuropathy according to a large prospective Dutch study was 47%, and the incidence in long survivors was even higher – 61% (Hilkens & van den Bent, 1997; van der Hoop et al., 1990).

The main prognostic factor for the severity of neuropathy is the cumulative dose of cisplatin. According to recent clinical data cisplatin-induced neuropathy is a strongly dose depended predominantly large fiber sensory PNP which develops after cumulative doses of 300 mg/m^2 or higher (Cersosimo, 1989; Ongerboer de Visser et al., 1985; van der Hoop et al, 1990). The single-dose intensity also seems relevant to the development of neuropathy (Cavaletti et al., 1992a).

Initial symptoms are usually numbness and paraesthesias in the stocking-and-glove distribution extending proximally with increasing cumulative dose, loss of tendon reflexes and a decrease in mainly thick fiber mediated sensory qualities such as vibration perception, fine touch perception and proprioception. This can lead to difficulties in small motor coordination. Neuropathy becomes markedly disabling at a cumulative dose of about 600 mg/m^2. The sense of joint position becomes impaired, resulting in profound sensory ataxia, including gait disturbances, loss of manual dexterity and becoming wheel-chair bound (Alberts & Noel, 1995). Motor power remains intact (Boogerd, 1995; Hilkens & van den Bent, 1997; Schattschneider et al., 2001). Decreased pain and temperature sensation are not prominent in clinical presentation. Pain is not a symptom of cisplatin-induced PNP (Krarup-Hansen et al., 1993; Roelofs et al., 1984). Some patients develop Lhermitte's sign or experience paraesthesias and electric shock sensation on stretching the arms or legs. This corresponds with electrophysiological and pathological findings of dorsal column involvement (Walsh et al., 1982).

The unambiguous information concerning the prognosis and possible predispositional factors of PNP has not been reported. The progrediental course can often be observed. Neuropathy worsens with further treatment and symptoms may begin or progress up to four months after cisplatin treatment has been discontinued (Alberts & Noel, 1995; Schattschneider, 2001). Recovery is usually incomplete and 30 to 50% of cases are irreversible even years after cessation of chemotherapy (Alberts & Noel, 1995; Strumberg et al., 2002).

Ototoxicity occurs almost as frequently as neuropathy and is considered to be due to the widespread loss of outer hair cells in the organ of Corti (Alberts & Noel, 1995). It is characterized by progressive hearing loss in the high frequency range over 4000 Hz (Smoorenburg et al., 1999). The clinical spectrum varies between otalgia, tinnitus, vestibular alterations and severe deafness (Schaefer et al., 1985). Although tinnitus is reversible, cisplatin-induced hearing loss is progressive and irreversible (Schweitzer, 1993).

The involvement of autonomic nerve fibers presenting mostly as a dysfunction of heart and orthostatic regulation has been occasionally reported (Rosenfeld & Broder, 1984).

Clinical signs of central nervous system involvement have also been reported (Highley et al., 1992; LoMonaco et al, 1992; Philip et al., 1991), including focal encephalopathy, epileptic seizures and cortical blindness. Ocular toxicity, including optic nerve degeneration and retinal infarcts, is a common complication of intracarotid cisplatin administration (Maiese et al., 1992).

1.3.2 Electrophysiological findings

Cisplatin-induced nerve conduction changes indicate the damage of large myelinated sensory fibers in both animal models and human studies. Typically, nerve conduction studies show absence or reduction up to 80-90% of the amplitude of the sensory action potentials, with only a mild to moderate decrease of sensory nerve conduction velocities (SNCV), and prolonged or absent H-reflexes, suggesting an axonal type of PNP (Daugaard et al., 1987; Krarup-Hansen et al., 1993; LoMonaco et al., 1992; Roefols et al., 1984; Thompson et al., 1984; Verdu et al., 1999).

Motor nerve conduction and electromyography (EMG) are usually normal or only mildly affected, indicating that the disturbance primarily involves sensory nerves (Alberts & Noel, 1995; Boogerd, 1995; Cerosimo, 1989; Hilkens & van den Bent, 1997). Somatosensory evoked potential studies showed slowing of conduction velocity along peripheral and central pathways after tibial nerve stimulation compatible with a toxic effect on the dorsal root ganglia cells causing "dying back" axonal degeneration of central and peripheral nerve fibers (Hansen et al., 1989; Krarup-Hansen et al., 1993).

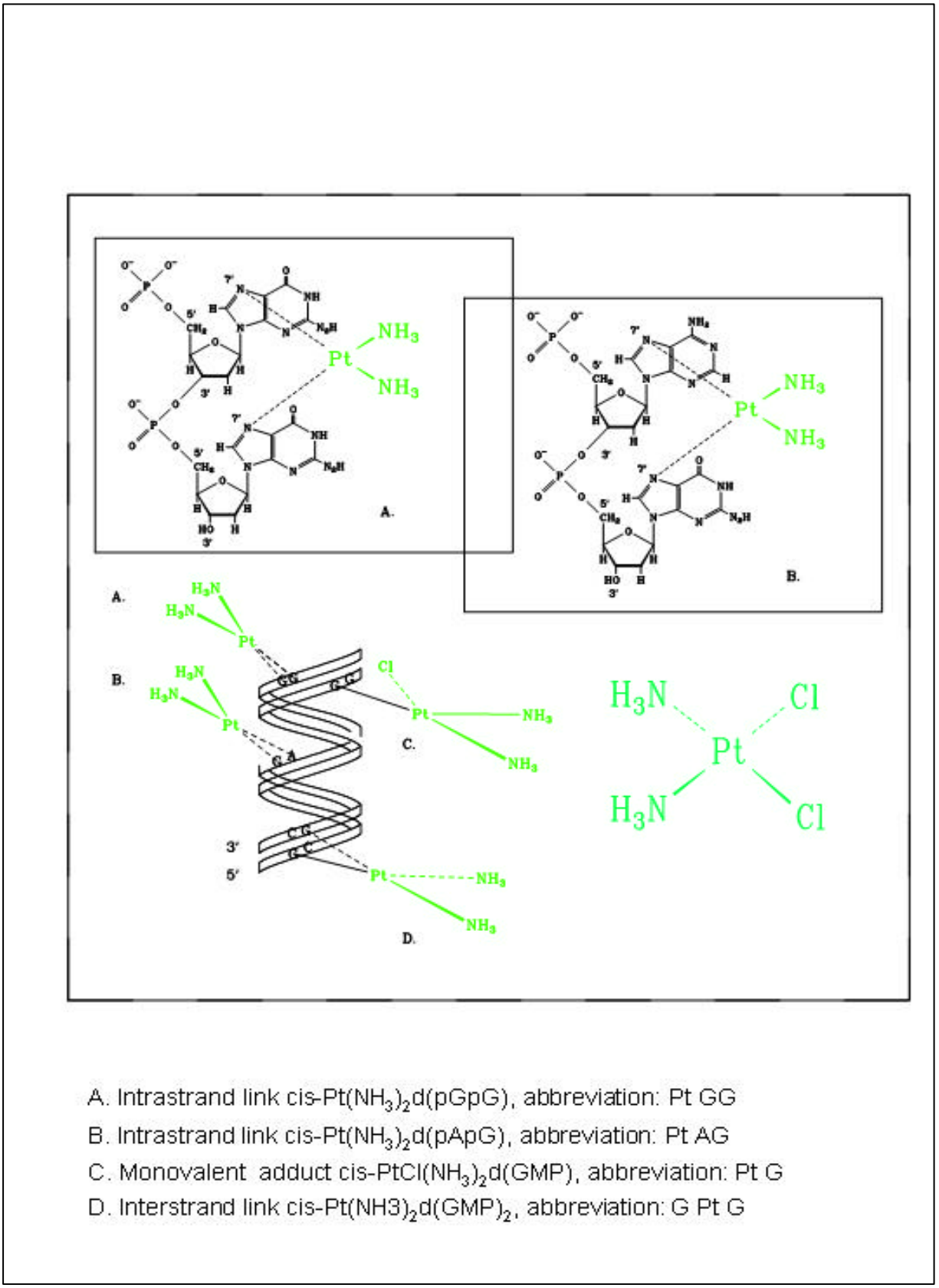
1.3.3 Morphopathological findings

Using classical histological and morphological techniques it has been shown that the clinical features of cisplatin-induced polyneuropathy result from predominant loss of large myelinated sensory fibers. Pathological examinations of sural nerve generally reveal axonal degeneration with a mild secondary segmental demyelination of large myelinated sensory fibers (Thompson et al., 1984; Walsh et al., 1982). Morphometric analysis has confirmed a disproportionate loss of large myelinated fibers (Gastaut & Pellissier, 1985). Experimental and autopsy studies further suggested sensory neurons and satellite cells to be the main targets of cisplatin neurotoxic effects (Cavaletti et al., 1992b; Riggs et al., 1988; Tomiwa et al.,1986). In rodents exposed to cisplatin early morphological alterations in DRG neurons were characterized by shrinkage of the cytoplasm, cell nucleus and particularly by reduction in the nucleolar size (Barajon et al., 1996; McKeage et al., 2001; Muller et al., 1990). Interestingly, comparative morphological analysis of DRG neurons and satellite cells in cisplatin treated rats showed rather satellite cells to have severe ultrastructural changes in the nucleus and cytoplasm, which were more prominent than in the corresponding neurons, suggesting that the satellite cells are also involved in the pathophysiology of cisplatin-induced PNP (Corsetti et al., 2000).

1.4 Antineoplastic activity and unwanted side effects as a consequence of Pt-DNA adduct formation

1.4.1 Interaction of Pt^{2+} - complexes with proteins and nuclear DNA

In the intracellular environment cisplatin is changed into Pt^{2+} hydroxo –complexes, which have a strong affinity to nucleophilic epitopes of proteins and nucleic acids. Pt-DNA modifications are assumed to represent by far the most important mediators of both the antineoplastic activity and the undesired toxic side effects of this potent anticancer drug. A very fast formation of intermediate ("early") monovalent Pt-DNA-adducts is followed by their conversion into "late" bivalent intra- and interstrand crosslinks within few hours (Bernges & Holler, 1991; Knox et al., 1986). Three different structures, linked at the N-7 position of adenines and guanines, have been identified as the major reaction products with DNA (figure 1): the intrastrand adducts cis-$Pt(NH_3)_2$d(pGpG), cis-$Pt(NH_3)_2$d(pApG) and cis-$Pt(NH_3)_2$d(pGpXpG), representing more than 95 % of DNA damage, and the interstrand crosslink cis-$Pt(NH_3)_2$d$(GMP)_2$ (Fichtinger-Schepman et al., 1985; Huang et al., 1995).



A. Intrastrand link cis-Pt(NH_3)$_2$d(pGpG), abbreviation: Pt GG
B. Intrastrand link cis-Pt(NH_3)$_2$d(pApG), abbreviation: Pt AG
C. Monovalent adduct cis-PtCl(NH_3)$_2$d(GMP), abbreviation: Pt G
D. Interstrand link cis-Pt(NH3)$_2$d(GMP)$_2$, abbreviation: G Pt G

Figure 1 Cisplatin-induced Pt-DNA adducts

1.4.2 Pharmacokinetic factors strongly influence adduct formation

The formation of adducts is influenced by several pharmacokinetic factors. Their regulation is genetically determined (figure 2).

Kidney function, organ perfusion (Schellens et al., 1996) and protective mechanisms like the blood-brain barrier control the extent of cellular drug exposure.

The intracellular platinum content of an individual cell depends on the balance between the drug import and export through the cellular membrane. While the existence of an active membrane import mechanism for cisplatin is still under discussion, there is at least one membrane protein known to perform an ATP-dependent transport of platinum complexes out of the cytoplasm into the extracellular space: MRP 2 (multidrug resistance protein) or cMOAT (canalicular multispecific organic anion transporter) – a membrane protein of the ABC (ATP binding cassette) transporter group. An augmented expression of MRP 2 can significantly increase the cellular platinum export rate and thus prevent the drug from reaching its molecular targets (Borst et al., 1999; Demeule et al., 1999; Klein et al., 1999; Matsunaga et al., 1998).

Cytoplasmatic detoxification by concurrent binding to glutathione or metallothionein is another pharmacokinetic factor which may influence the final nuclear DNA platination level. (Chao, 1996; Ishikawa & Ali-Osman, 1993; Meijer et al., 1992 & 2000; Pattanaik et al., 1992).

1.4.3 Pt-DNA adducts are substrates for the DNA repair machinery

Cells are equipped with several protective mechanisms of DNA repair which are required for maintaining the integrity of the genome exposed to environmental or endogenous DNA damaging agents. Cisplatin-induced DNA adducts are known to be eliminated through a pathway, called nucleotide excision repair (NER) (Crul et al., 1997; de Laat et al., 1999; Sancar, 1996; van Steeg et al., 2000). A set of several proteins is responsible for adduct recognition, unwinding of DNA, incision, excision of adduct bearing DNA fragments and at last for DNA synthesis and ligation (Figure 2). The lack of at least one functional NER component, like the damage recognizing XPA protein, can result in an extreme sensitivity of cells to the acute toxicity of cisplatin (Asahina et al., 1994; Dabholkar. et al 1994; Dijt et al., 1988; States & Reed 1996). By measuring the *in vivo* Pt-DNA adduct repair kinetics in various cell types, Liedert has demonstrated in an experimental model of XPA-deficient mice, that such hypersensitivity was due to an increased formation and / or reduced repair of specific Pt-DNA adducts like Pt-(GG) intrastrand crosslinks (Liedert, 2001).

1.4.4 The tolerance to Pt-DNA adducts determines the cellular sensitivity to platinum based chemotherapy

The degree of tolerance to persisting (unrepaired) Pt-DNA lesions determines the fate of each cell, which may be survival or apoptosis (Figure 2; Crul et al., 1997; Fajac et al., 1996; Ormrod et al., 1994 & 1996; Sorenson et al., 1990). Pt-DNA adducts hinder replication and transcription and recruit proteins like mismatch repair proteins (Branch et al., 2000; Lage & Dietel, 1999; Samimi et al., 2000) which directly induce cell death, activating p53 or p73 dependent pathways (Hawn et al., 1995; Li, 1999). The relative contribution of different mechanisms to the antineoplastic activity on the one hand and to the unwanted side effects on the other is far from being understood. Furthermore, the relative toxicity of the structurally different DNA adducts is also discussed controversially.

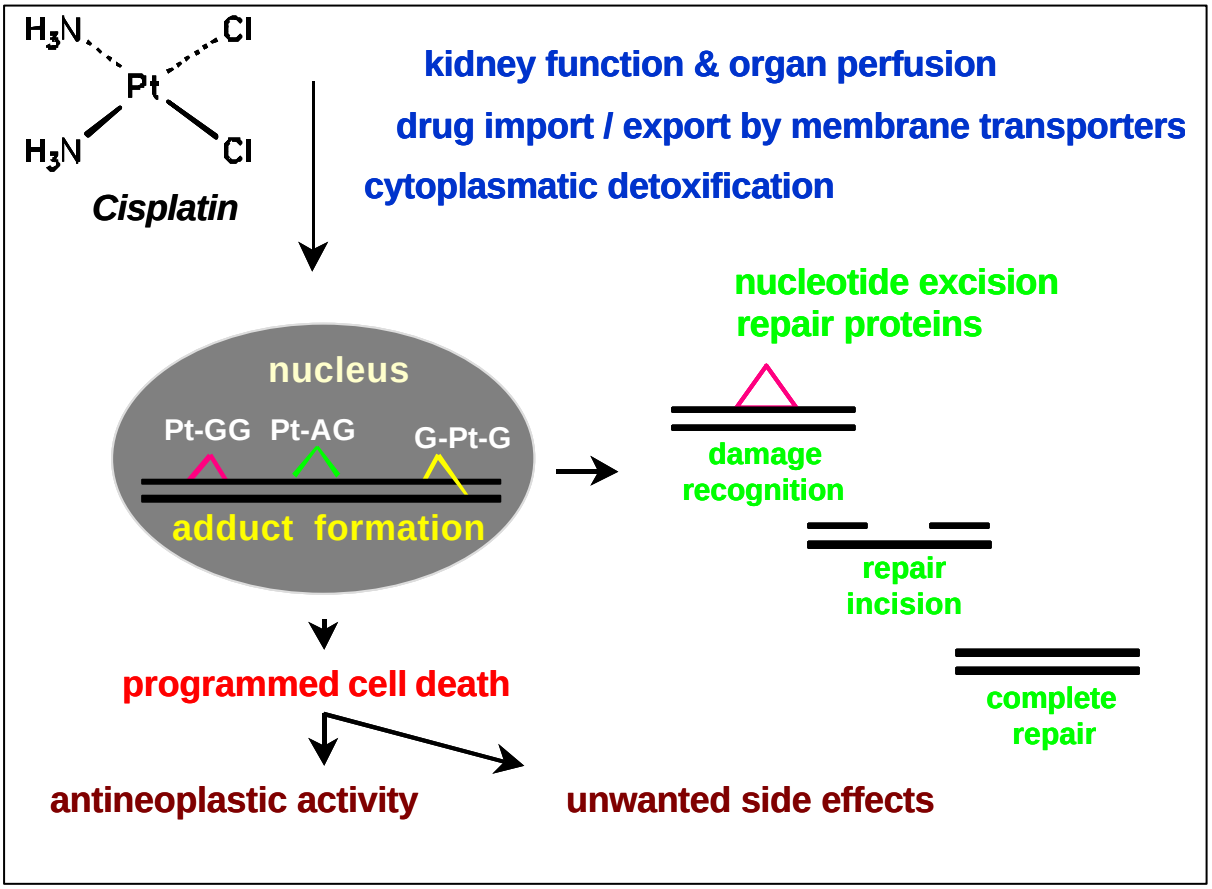


Figure 2 Antineoplastic activity and unwanted side effects of cisplatin depend on formation, repair and tolerance to Pt-DNA adducts

1.4.5 Formation, repair and tolerance to Pt-DNA adducts are cell-type specific and interindividually variable

Pt-DNA-adduct accumulation and elimination rates as well as the degree of tolerance to such DNA damage are known to be cell type specific (Fichtinger-Schepman et al., 1989; Johnsson et al., 1995; Mustonen et al., 1989; Terheggen et al., 1987). This may partly explain the restriction of unwanted side effects to some tissues. Due to the differences in the genetic background, prominent interindividual heterogeneities in adduct levels can also be observed (Meijer et al., 1999), leading to a broad range of both the therapeutic efficiency and toxicity to normal cells.

1.4.6 Pathophysiological models for platinum induced neurotoxicity

The contribution of different cisplatin-induced toxic effects to neuronal damage is unclear. Microtubuli cross-linking caused by platinum protein interaction might lead to the impairment of fast axonal transport (Boekelheide et al., 1992; Gao et al., 1995; Russell et al., 1995).

Both autopsy and animal studies have demonstrated that cisplatin-induced neurotoxicity is associated with platinum accumulation in the DRG whereas the levels of DNA platination in the brain and spinal cord proved to be significantly lower (Gregg et al., 1992; Poirier et al., 1992 & 1993; Terheggen et al., 1989; Thompson et al., 1984). As DRG cells are located outside the central nervous system (CNS), they are not protected by the blood-brain barrier (BBB), which obviously represents an effective defense mechanism against cisplatin exposure. Central neurotoxicity can be observed in the situations where the BBB is weakened, e.g. in the cases of brain tumors or by high dose mannitol co administration (Fountzilas et al., 1991).

The primary damage of DRG cells explains the sensory type of cisplatin-induced PNP with the preservation of motor nerve fibers.

Hence, the molecular mechanisms of neurotoxicity might be related to DNA damage in the DRG sensory neurons and satellite cells by formation of Pt-DNA adducts.

Some studies point out, that neurotoxicity is mainly due to the cisplatin-induced apoptosis of DRG neurons (Gill & Windebank, 1998; Fischer et al., 2001; McDonald & Windebank, 2002). Other investigations focus on DRG satellite and Schwann cells, which have been shown to develop severe ultrastructural changes after cisplatin exposure (Corsetti et al., 2000; Sugimoto et al., 2001; Terheggen et al., 1989). As far as the satellite cells are involved in several essential neuronal functions such as intra- and extraneuronal transport and the trophic function, it seems quite plausible that at least partially the loss of DRG neurons can be secondary to cisplatin-induced glial damage.

1.5 Analytic amenability of Pt-DNA adducts

In order to improve the knowledge about the molecular mechanisms of platinum related cytotoxicity, numerous analytical attempts have been made within the past 20 years to detect and quantify Pt-DNA adducts in experimental systems and in clinical specimens. Among these methods are spectroscopic procedures like nuclear magnetic resonance (NMR) (Fichtinger-Schepman et al. 1982), atom absorption spectrometry (AAS) (Reed et al., 1990a, b; & 1993) or inductively coupled plasma mass spectroscopy (ICP-MS) (Bonetti et al., 1996), ^{32}P-postlabeling techniques (Blommaert & Saris 1995; Farah et al. 2000; Pluim et al. 1999; Welters et al. 1997 & 1999a, b), immuno-analytical assays based on polyclonal or monoclonal antibodies (Chao et al. 1994; Blommaert et al. 1996; Fichtinger-Schepman et al. 1985 & 1987; Meijer et al., 1997 & 1999; Poirier et al. 1982; Sundquist et al. 1987; Terheggen et al. 1987 & 1991) or PCR-based techniques (Grimaldi et al., 1994; Bingham et al., 1996;). Further progress in understanding clinical resistance and unwanted side effects was hampered, because some of these methods proved to be either not sensitive enough for the measurement at the relevant DNA platination levels (NMR, PCR), or to be unable to distinguish between specific lesions (AAS, ICP-MS, ELISA, immunohistochemistry), or could not work at the level of single cells (all methods except immunohistochemistry).

The recent development of an “Immuno-Cytological Assay” (ICA), based on anti-(Pt-DNA adduct) MABs allows one to quantitate the defined Pt-DNA adducts at a single cell level and to realize molecular dosimetry of these lesions (Liedert 2001).

1.6 Aim of the study and future perspectives

It is unclear, whether the enhanced adduct formation, insufficient DNA repair or poor adduct tolerance is responsible for the sensitivity of nervous tissue to platinum based chemotherapy. It also remains to clarify whether glial cells or rather neurons are prone to functional damage by cisplatin.

This report demonstrates, that adduct-specific single cell dosimetry of Pt-DNA adducts by means of the immunocytological assay is suitable to elucidate these questions. Investigations using XPA- and XPC-knockout mice give a detailed insight into the mechanism of the NER adduct recognition step and its importance concerning neurotoxicity.

In the future, single cell quantification of Pt-DNA adducts might be a powerful tool for the development and testing of chemoprotective substances, which should meet contradictory demands: effectively prevent patients from the therapy induced neuronal damage without influencing the antineoplastic activity of cisplatin (Awada & Piccart, 2000; Babu et al., 1999; Hanada et al., 1999; Hartmann et al., 1999; Korst et al., 1998, Links & Lewis, 1999; Osman et al., 2000).

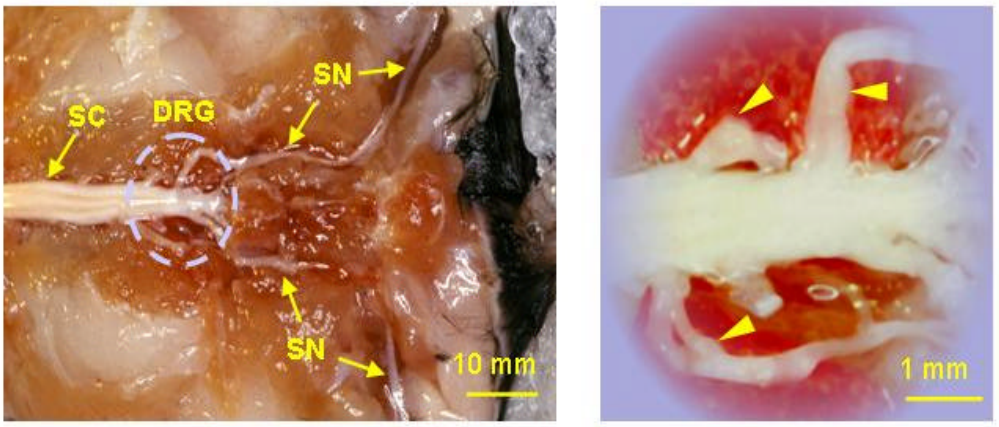
2 RESULTS & COMMENTS

2.1 Optimization of the immuno-cytological assay (ICA) for the measurement of Pt-DNA adducts in nervous tissues

The method of “Immuno-Cytological Assay” (ICA), firstly established in the Institute of Cell Biology, University of Essen, by Seiler et al. (1993) and Thomale et al. (1994), has been recently developed by Liedert (2001) to a highly sensitive and specific monoclonal antibody-based Pt-DNA-adduct quantification technique, which basically allows the measurement of these DNA lesions at the single cell level both within cultured cells and in biopsy material.
To enable the cell type-specific molecular dosimetry of cisplatin-induced DNA lesions within the structures of the central and peripheral nervous system in the *in vivo* mouse model, this method has been technically optimized and supplied with the immunohistochemical cell type identification step.

2.1.1 Preparation of nervous tissue samples for ICA procedure

Traditional ways of tissue sample preparation for immunohistochemical procedures, like glutaraldehyde – paraformaldehyde fixation with paraffin embedding or perfusion of organs with fixative solutions in situ, significantly decrease permeability of cellular and nuclear membranes for the antibodies designed against intranuclear antigens. Therefore, to obtain the optimal penetration of anti-(Pt-DNA) MABs into the cell nuclei, the use of microtome sections from native frozen tissue specimens is required. This method generally ensures good preservation of cell structure and antigens (Harlow & Lane, 1988). However, the principal disadvantage of preparing the samples from non-fixed nervous tissues is that the nervous cells are extremely sensitive to acute postmortal anoxia and therefore are prone to early development of postmortal structural changes with the loss of target antigens. Thus, several peculiarities have been invented to adjust the preparation technique for gentle tissues of DRG and the spinal cord and to shorten the time window from the death point until the deep freezing of samples.
To prevent the nervous structures from early enzyme-induced postmortal morphological damage, the carcass was kept in ice during the organ dissection and the exposed organs were constantly rinsed with PBS (0.01M, pH 7.4) precooled to 4°C.
A midline dorsal longitudinal incision over the spine and an expanded microlaminectomy served as quick and non-traumatic way to expose the lumbar DRGs and the lumbar part of the spinal cord (figure 3).
Freshly dissected tissue samples were immediately submerged into “Tissue-Tek O.C.T. Compound” embedding medium and deep frozen in liquid nitrogen. Frozen tissue blocks were placed into precooled freezing vials and stored at -80°C.



A B

A. Lumbar part of the spinal cord (SC) with lumbar DRG at both sides is gently exposed by microlaminectomy; both sciatic nerves (SN) are exposed between muscles and pelvic bones. The exposed nervous tissues are constantly rinsed with precooled (4°C) PBS

B. Lumbar DRG (arrow head) with radices at both sides are exposed and dissected microscopically

Figure 3 Preparation of nervous tissue samples for the ICA procedure

2.1.2 Immuno-histochemical identification of different neuronal cell types

The cell sensitivity to cisplatin as well as the Pt-DNA-adduct accumulation rates are known to be tissue and cell type specific (1.4.5). However, it was to clarify, whether there are any differences in adduct accumulation and repair rates between the neuronal and glial cells as well as between the central and peripheral nervous system cells and whether this might correlate with the type of cisplatin-induced neurological deficit. Therefore, it proved to be essential to quantify the Pt-DNA-adduct levels separately for neuronal and glial cells of the DRG or spinal cord as well as for the Schwann cells of the sciatic nerve. A double-labeling experiment, where cell type specific antigens and the Pt-DNA-adducts are marked with two different detection reagents in the same specimen, should obviously be the method of choice to achieve this goal. The obstacle one has to overcome is that the strong proteolytic cleavage of cytoplasmic and nuclear proteins, which could serve as type specific antigens, is required for optimal immunostaining of Pt-DNA-adducts. The solution of this problem is performing the cell type identification and the adduct immunostaining within the same tissue section sequentially as two independent steps of one assay, where at the first step the cell types are identified immuno-histochemically and this information is stored as a digital image, which is then used to identify cell types retrospectively after the Pt-DNA-adducts have been labeled in the second step.

The “NeuroTrace™ 530/615 red fluorescent Nissl Stain” was used to identify the neurons within the tissue sections of DRG or spinal cord. Glial cells of the spinal cord, DRG or sciatic nerve were stained with anti-CNPase MABs, secondary labeled with fluorochrome (“ALEXA FLUOR 488”). The nuclear DNA was counterstained with DAPI. The cell type identification was performed as analysis of digital images taken from the slides by means of a laser scan microscope. The neurons were determined by red, glial cells by green fluorescence, the cell nuclei – by DAPI (blue) fluorescence (figure 4). The images were stored electronically.

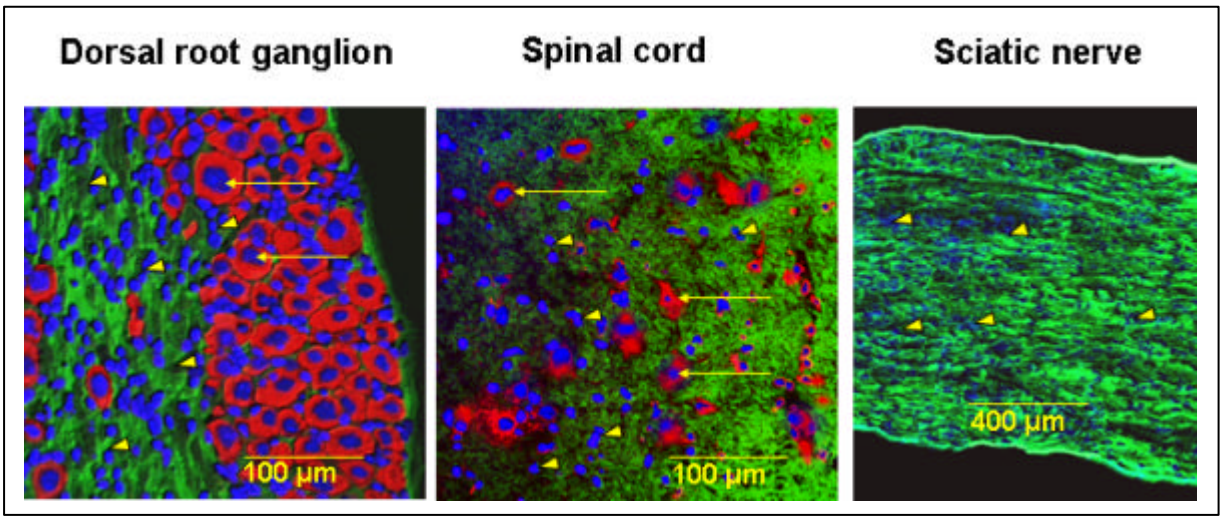


Figure 4 Immunohistochemical identification of different neuronal cell types: arrows – neurons; arrow heads – glial cells

Dithioeritritol solution in glycerol and polyvinyl alcohol (4.8.2) was used as a special water soluble mounting medium for the microscopy during the cell type identification to enable performing the adduct-specific immunostaining of the same probe thereafter.

2.1.3 Immunostaining of specific Pt-DNA adducts

After the neuronal cell types have been identified, the same probes were stained for Pt-DNA adducts according to the ICA protocol, adjusted for different nervous tissues.

2.1.3.1 Fixation, alkaline permeabilisation and proteolytic cleavage

Several fixation and denaturation techniques have been tested to achieve an optimal penetration of the antibodies through the highly myelinated membranes and to provide a good access of MABs to the intranuclear epitopes. A combination of Triton X-100 and alkaline permeabilisation was found as the best variant for the spinal cord and sciatic nerve. Compared to formamide fixation, microwave-denaturation or alkaline treatment alone this procedure provided suitable adduct specific nuclear staining with significantly lower background signal.

For the less myelinated DRG tissue sections, however, a single alkaline permeabilisation proved to be sufficient to obtain good immunostaining results.

The proteolysis of cytoplasmic and nuclear proteins with pepsin and proteinase K helped to access the antigen determinants and at the same time reduced the undesired non-specific antibody binding. Although the intensity of proteolytic cleavage, if applied to a nervous tissue section, has to be rather strong, the morphological integrity of cell nuclei should largely be preserved, as it is absolutely required for the single cell based adduct quantification. Therefore, the optimal enzyme concentrations and digestion conditions were tested and adjusted individually for each tissue type (4.8.3).

2.1.3.2 Sandwich immunostaining and DNA counterstaining

Cisplatin-induced bivalent Pt-DNA adducts were marked with anti-(Pt-GG) MABs and secondary labeled with fluorochrome.

The sensitivity of the adduct-specific immunostaining was significantly improved by applying a cascade of several fluorochrome-labeled secondary antibodies for the detection of Pt-DNA – anti-(Pt-DNA) immune complexes (Figures 5, 6). Such “sandwich” staining technique increases the rate of the fluorescence emission and therefore allows the precise visualization and quantification even of relatively low amounts of cisplatin-induced DNA lesions. To minimize the background signal, the incubation times of the secondary antibodies were reduced, 0.05% Tween 20 solution was applied for additional wash after each step and the duration of washes was increased.

DNA counterstaining with DAPI was used to identify the position of cell nuclei and to perform the calculation of relative adduct levels corrected for actual nuclear DNA content of each cell.

2.1.3.3 Quantification of relative adduct levels in well defined neuronal cells by digital cytometry

Detection and quantitative analysis of Pt-DNA-adducts were performed by means of the fluorescence-microscope coupled digital image analysis system “ACAS”. The cell nuclei were localized by DAPI-DNA fluorescence and different cell types were marked manually using the digital images obtained at the cell type identification step (figure 5). Adduct levels in nuclear DNA of individual cells were calculated by normalizing antibody-derived fluorescence signals to the corresponding DNA content of the same cell (in order to correct for the possible DNA loss) and expressed as relative units (RU). These data were assigned to specific cell types as determined by immuno-histochemistry (2.1.2) Thus, finally, the relative adduct levels corrected for nuclear DNA content were used as quantitative parameters (figure 6). The mean adduct amounts of at least 200 cells per each cell type were used as values.

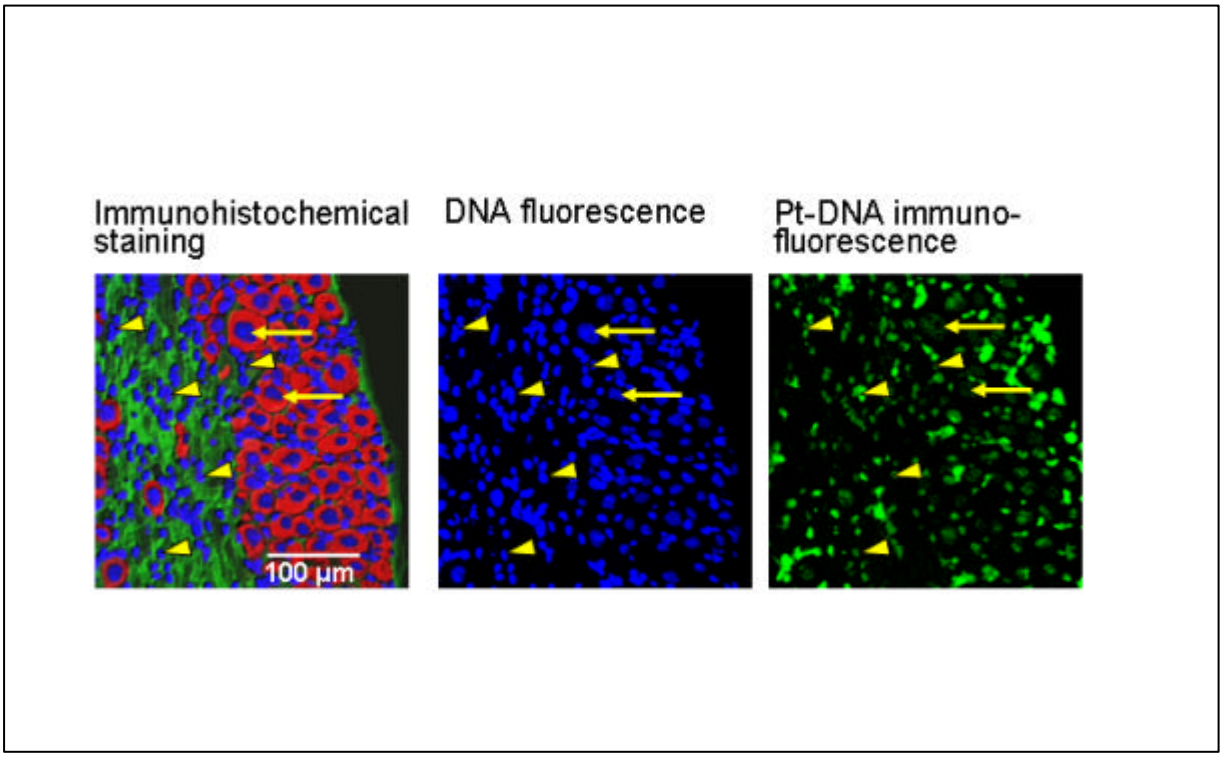
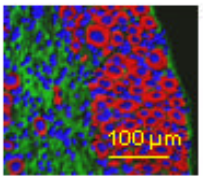


Figure 5 Detection of Pt-DNA adducts in well defined cell types of the murine nervous system at the single cell level: arrows – DRG neurons; arrow heads – satellite cells (DRG frozen tissue section, 24h after single cisplatin treatment)

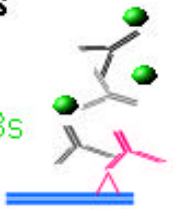
- ***in vivo* Cisplatin exposure**
- **Frozen tissue sections**
  - DRG, spinal cord, sciatic nerve

- **Immunohistochemical cell type identification**
  - Neurons
  - glial cells
  - cell nuclei

  } Digital image analysis



- **Immunostaining for specific Pt-DNA adducts**
  - anti-(PT-DNA) MAB
  - signal cascade of fluorochrome-labelled SABs
  - DNA counterstaining



- **Quantification of adducts at the single cell level**
  - Localisation of cell nuclei by DNA fluorescence
  - Measurement of DNA and immunofluorescence
  - manual marking of different cell types
  - Relative adduct levels corrected for nuclear DNA content
  - Results as a mean adduct amount of at least 200 cells for each cell type

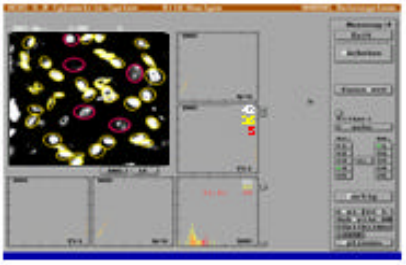
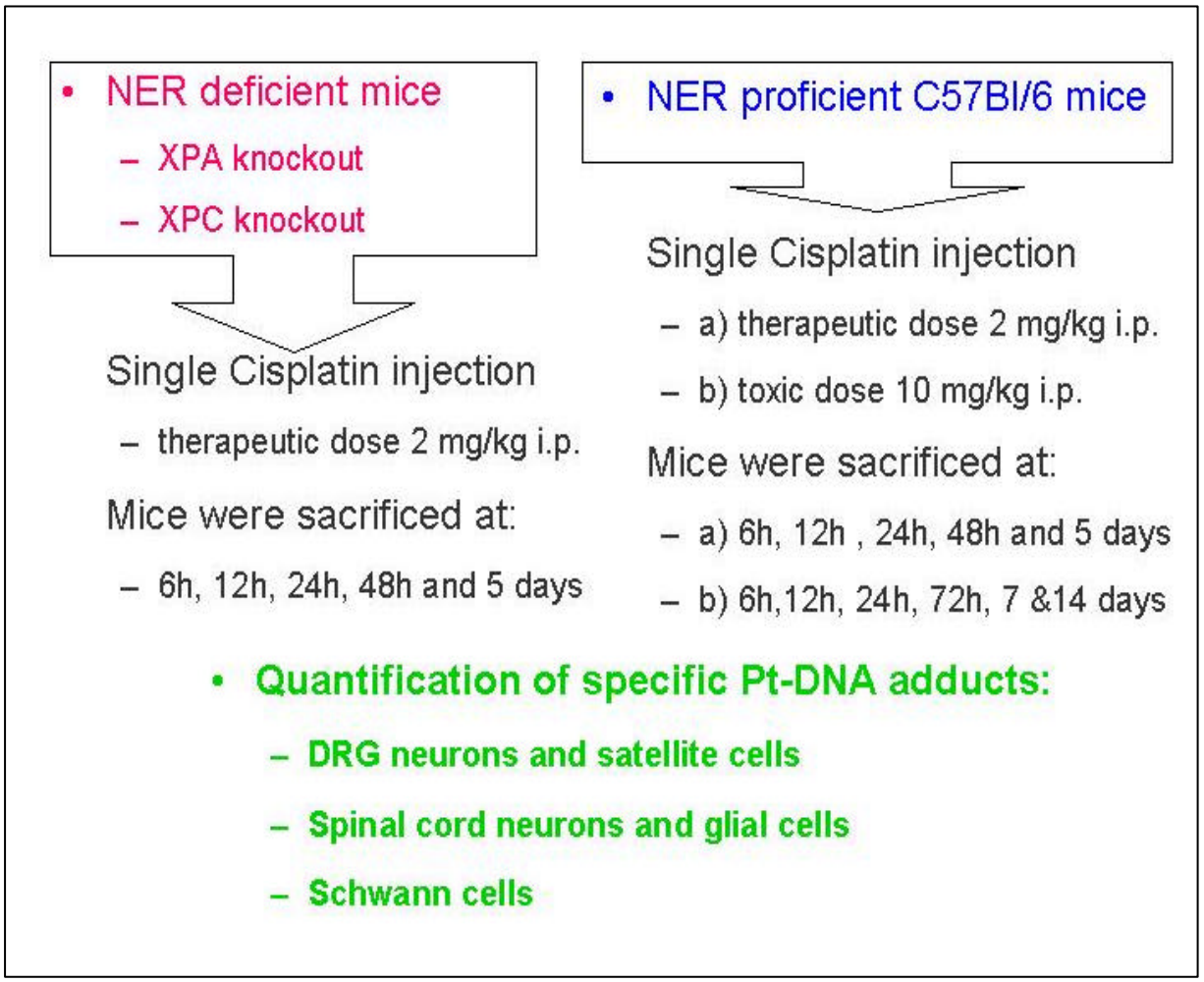


Figure 6 Immuno-Cytological Assay (ICA) for the quantification of cisplatin-induced DNA adducts at the single cell level (overview)

2.2 Formation and repair of specific Pt-DNA lesions in the nervous tissue of wild type and DNA repair deficient mice

2.2.1 Study design



2.2.2 Cell type-specific accumulation and repair of Pt-DNA adducts in nervous tissue of WT mice

C57Bl/6 mice were used as wild type (WT), DNA repair proficient animals to establish the murine model of acute cisplatin-induced neurotoxicity. Cisplatin was administered as a single i.p. injection in the dosages of 2 or 10 mg/kg. The low dose was used as an equivalent to the clinical therapeutic dose, the higher dose represented 70 % of the mouse LD_{50} (Connors et al., 1972) and corresponded to the neurotoxic dose in humans (van der Hoop et al, 1990). The animals were sacrificed according to the schedules described above (2.2.1). Cell type specific accumulation and repair of cisplatin-induced DNA lesions in the central and peripheral nervous system were analyzed in the ICA. Animals both under the low or high dose treatment showed no signs of vital functions limitation throughout the experiments.

After single dose administration of cisplatin (2 mg/kg) Pt-DNA lesions were detectable in all cell types investigated. Accumulation of adducts in DRG neurons and satellite cells (outside the blood-brain barrier [BBB]) was significantly higher than in the same cell types of the spinal cord. Further, the extent of adduct burden was higher in DRG satellite (0.49 ± 0.019 RU) or spinal cord glial cells (0.28 ± 0.009 RU) than in the neurons of the same anatomical structures (0.34 ± 0.013 RU for DRG neurons, 0.23 ± 0.009 RU for spinal cord neurons, $p < 0.01$; figure 7).

Interestingly, the lowest adduct levels were observed in the Schwann cells of the sciatic nerve, being about 3.7 times lower than in satellite cells (0.13 ± 0.005 RU versus 0.49 ± 0.019 RU, $p < 0.01$) and even lower than in the glial cells of the spinal cord (figure 7).

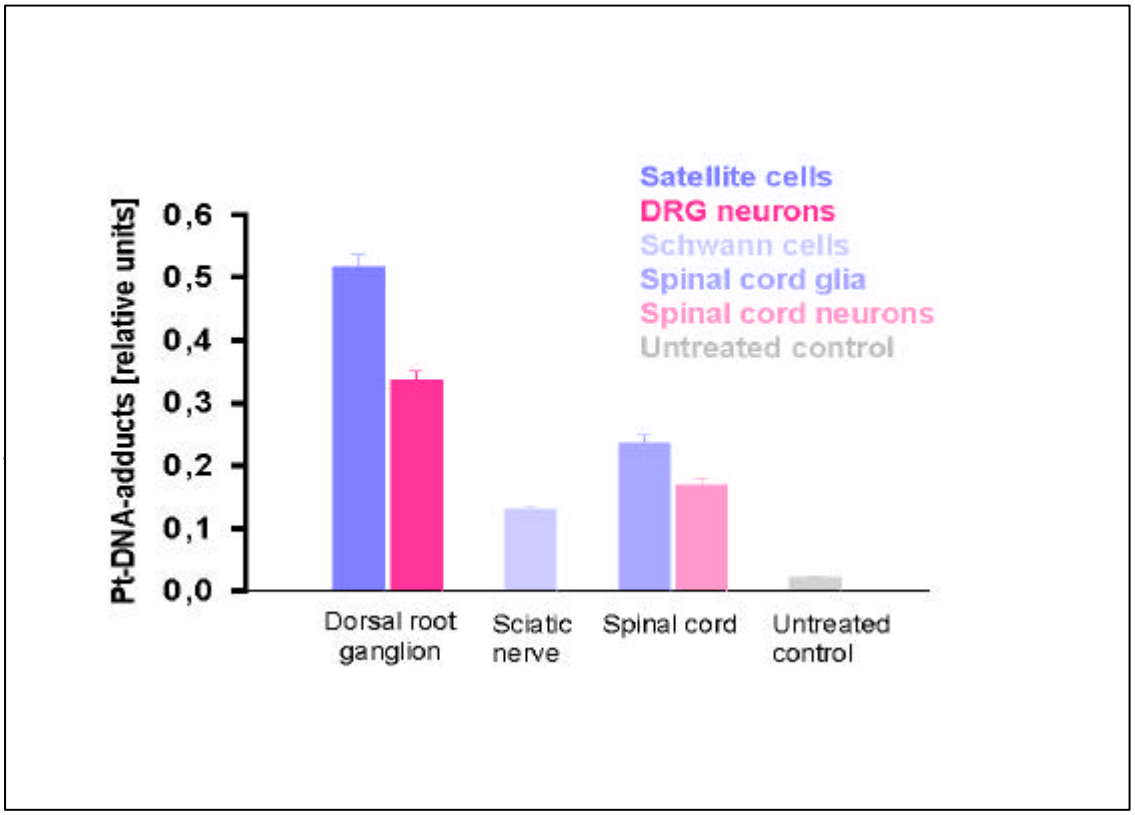


Figure 7 Pt-DNA adduct accumulation in different cell types of the murine nervous system (WT mouse, 24h after cisplatin treatment 2 mg/kg)

Adduct accumulation was dose dependent: 2.08 ± 0.092 RU and 0.66 ± 0.027 RU in DRG satellite cells and DRG neurons following single administration of 10mg/kg cisplatin; 0.49 ± 0.019 RU and 0.34 ± 0.013 RU in DRG satellite cells and DRG neurons following single administration of 2 mg/kg cisplatin, $p < 0.01$ (figure 8). The major accumulation profiles remained unchanged under the high-dose schedule: the maximal adduct levels were observed in the DRG cells; satellite and spinal cord glial cells contained more DNA lesions than corresponding neurons. However, the toxic dose treatment led, probably due to the better blood-brain barrier penetration, to an increased adduct formation within the CNS cells, so that the adduct levels in spinal cord glial cells even exceeded those of the DRG neurons (figure 8).

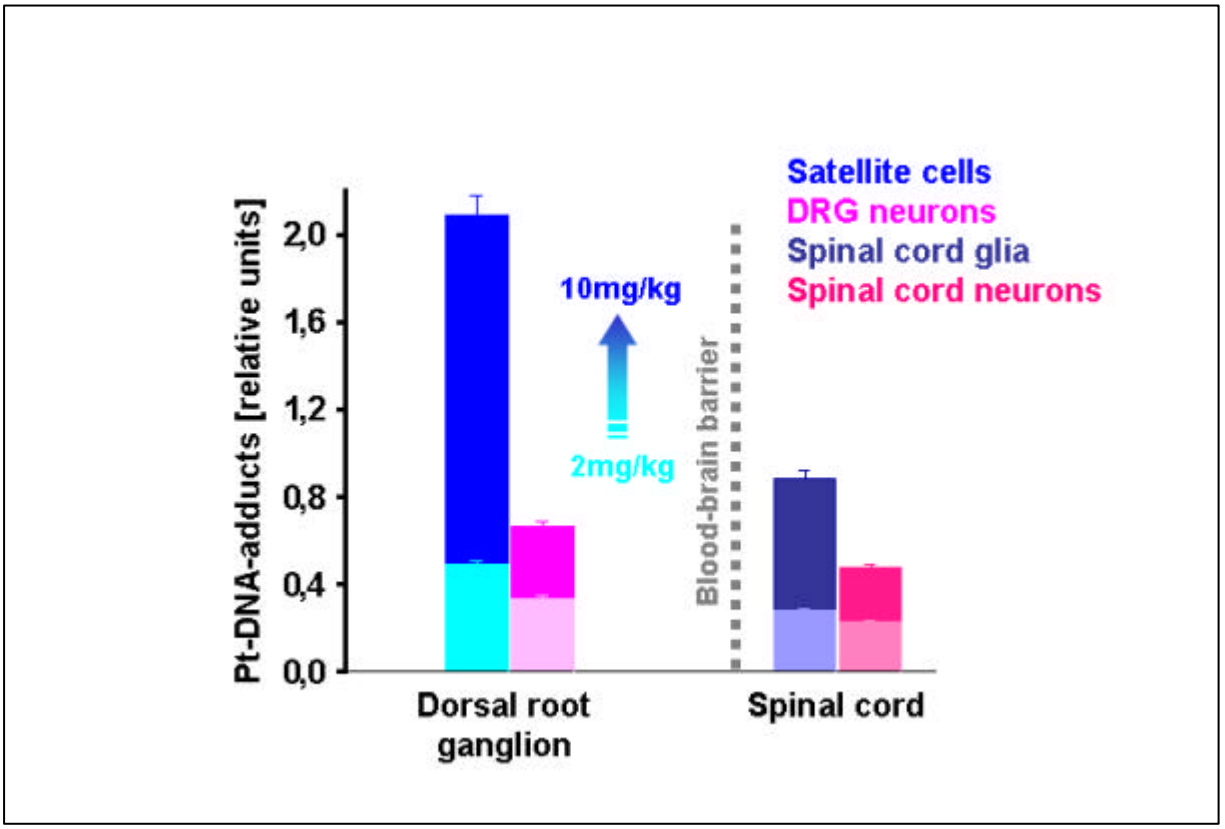


Figure 8 Pt-DNA adduct accumulation in the central and peripheral nervous system under the low dose (2mg/kg) and high dose (10mg/kg) cisplatin administration (WT mouse, 24h after single treatment)

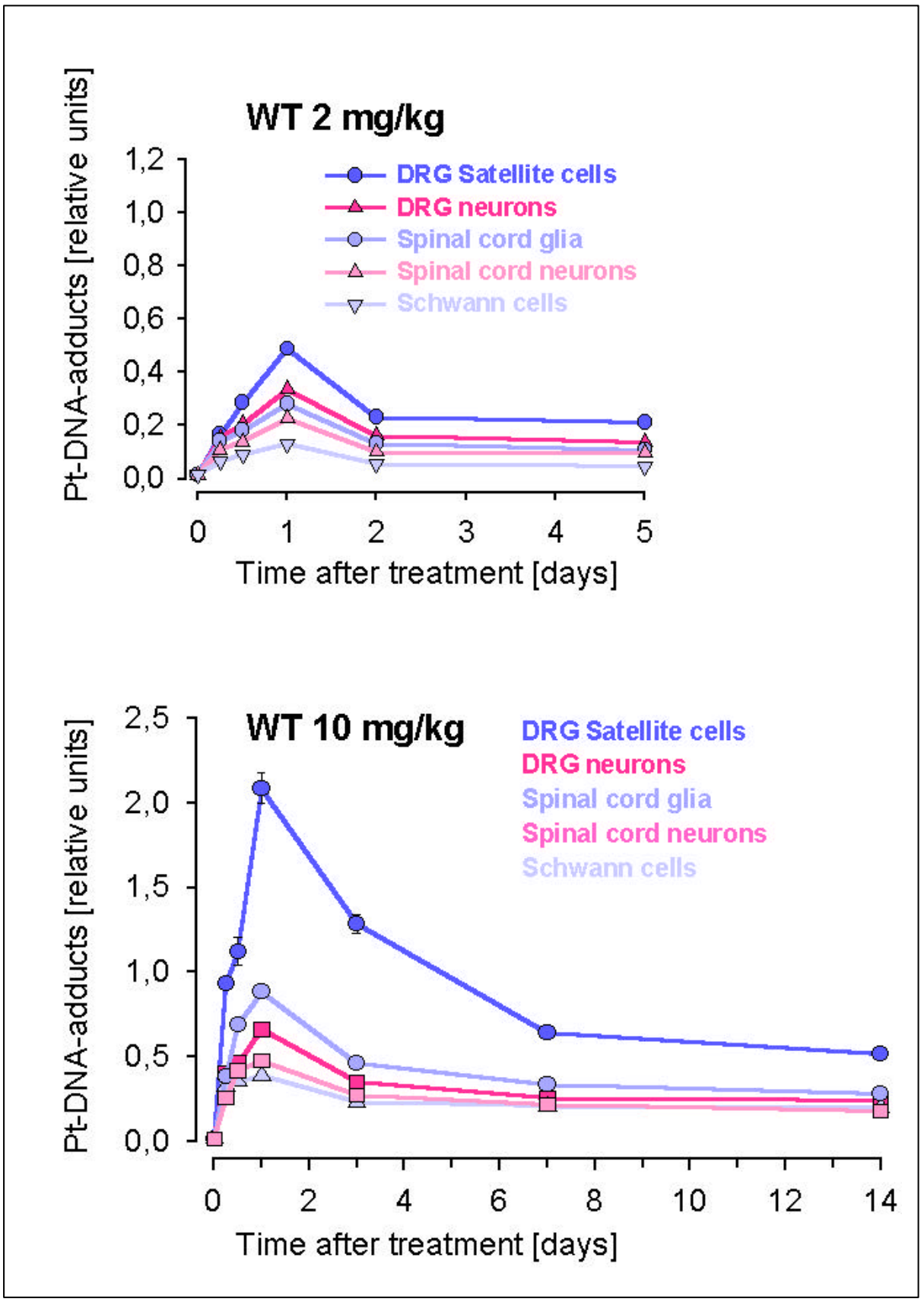


Figure 9 Pt-DNA adduct formation - repair kinetic in different neuronal cell types of NER proficient C57Bl/6 mice

The maximum adduct levels were reached in all investigated cell types 24 hours after treatment. Possible reasons for such relatively slow increase in Pt-DNA adduct amount could be gradual mobilization of the drug initially bound by plasma proteins, as well as the time, necessary for transformation of intermediate monovalent platination products into bivalent adducts (Liedert, 2001). The proportions of adduct accumulation between the cell types remained unchanged at all observed time points, though the quantitative differences became less prominent after repair phase. The adduct elimination profiles representing the efficiency of DNA repair were similar in different cell types and under different dose schedules: up to 70% of lesions were removed in the course of the intensive repair phase, whereas the rest of adducts persisted as a plateau-level, which decreased slowly till the latest time point after application (5 or 14 days respectively) and represented approx. 30% of the initial burden. The duration of intensive repair phase was dose dependent: 24h under the low dose and 48h under the high dose cisplatin administration with the exception for satellite cells, where the intensive repair phase after high dose treatment was prolonged (due to the high initial adduct burden) up to 6 days following the maximal level (figure 9).

2.2.3 Pt-DNA adduct accumulation - repair kinetic in NER-deficient mice

To investigate the role of DNA repair in the pathophysiology of cisplatin-induced neurotoxicity two NER-deficient mouse strains – XPA- and XPC-knockout – were treated with therapeutic dose of cisplatin (2 mg/kg) and sacrificed according to the described schedule (2.2.1). The results obtained in the ICA were compared with those of WT mice received the same treatment.

As previously observed in WT mice, adduct levels peaked 24h after treatment. Both NER deficient strains showed about two-fold higher Pt-DNA adduct accumulation in all cell types, which is already visible as significantly higher immunofluorescence intensity (figure 10).

The cell type specific accumulation patterns reproduced those of WT mice with high adduct accumulation in satellite cells and neurons of the DRG (0.92 $\pm$ 0.035 RU in DRG satellite cells of $XPA^{-/-}$ mice and 0.92 $\pm$ 0.048 RU in those of $XPC^{-/-}$ mice; 0.59 $\pm$ 0.034 RU in DRG neurons of $XPA^{-/-}$ mice and 0.58 $\pm$ 0.039 RU in those of $XPC^{-/-}$ mice) and less accumulation in glial cells and neurons of the spinal cord (0.42 $\pm$ 0.018 RU and 0.45 $\pm$ 0.020 RU in spinal cord glial cells of $XPA^{-/-}$ mice and $XPC^{-/-}$ mice; 0.34 $\pm$ 0.015 RU and 0.30 $\pm$ 0.014 RU in spinal cord neurons of $XPA^{-/-}$ mice and $XPC^{-/-}$ mice respectively, $p < 0.01$; figure 11).

The Schwann cells of the sciatic nerves showed again the lowest adduct formation rates compared to the other glial cell types (0.30 ± 0.018 RU and 0.32 ± 0.022 RU in the Schwann cells of $XPA^{-/-}$ mice and $XPC^{-/-}$ mice respectively; figure 11).

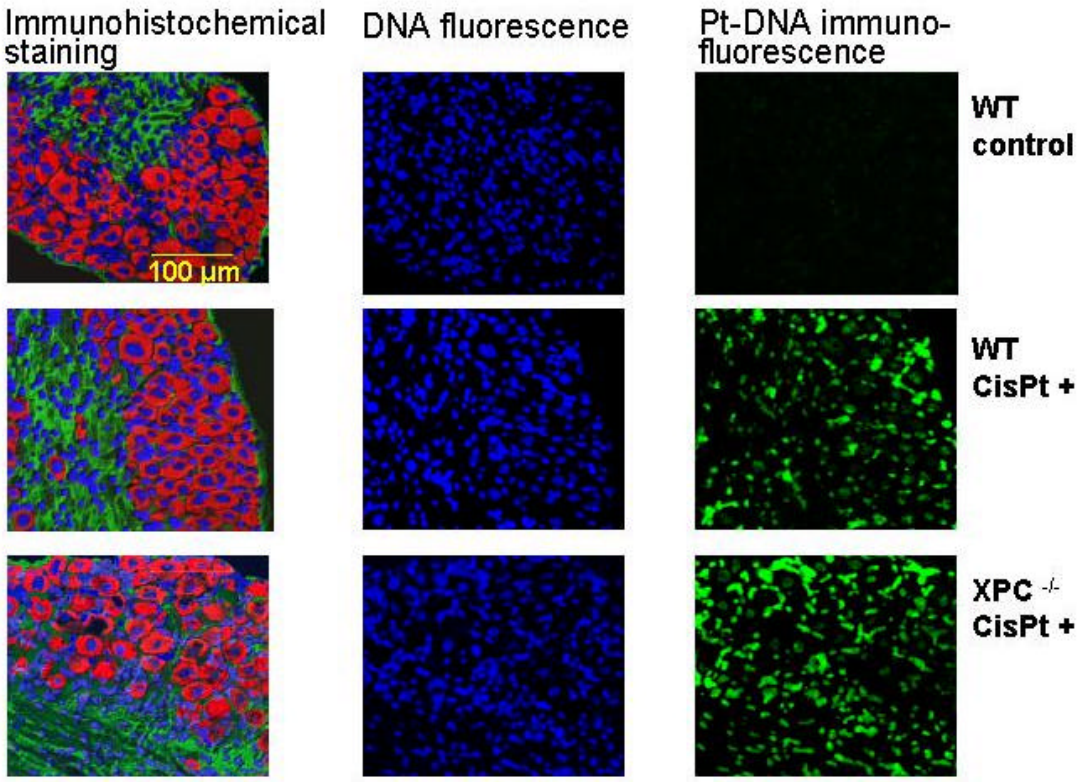


Figure 10 Detection of Pt-DNA adducts in DRG neurons and satellite cells of NER proficient and NER deficient mouse 24h after cisplatin treatment, 2mg/kg i.p.

Although the absence of active XPA protein led to a sharp increase in accumulation of cisplatin-induced DNA crosslinks, some removal of these lesions still could be observed in all the investigated cell types (30% by day 2), indicating that both neuronal and glial cells with this defect were nevertheless capable of limited DNA repair (figure 11).

The nervous tissue cells lacking XPC function were in contrast to WT and $XPA^{-/-}$ cells completely unable to eliminate the Pt-DNA crosslinks from the genome and were characterized by long term persistence of adducts (figure 11). These findings allow the suggestion that the XPC protein but not XPA is essential for the recognition and repair processing of bivalent Pt-DNA adducts like Pt-(GG).

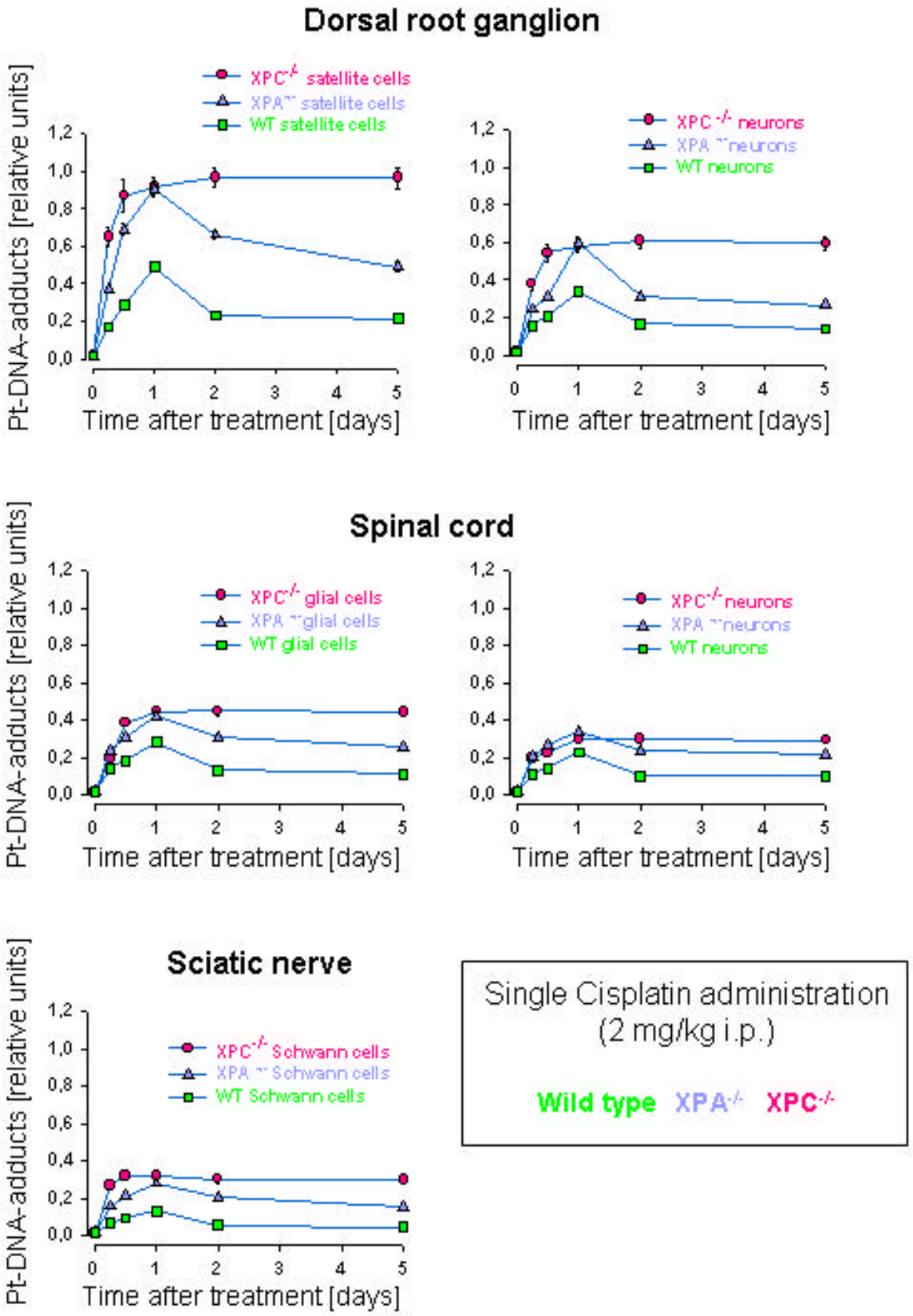


Figure 11 Platinum-DNA adduct repair kinetics in wild type and DNA repair deficient mice

2.2.4 Enhanced acute cisplatin neurotoxicity in XPA- and XPC-knockout mice

The XPA-knockout mice were shown to be extremely sensitive to the toxic effects of cisplatin and morphological signs of tubular kidney damage could be observed already 5 days after a single administration of 2 mg/kg (Liedert, 2001). In the present experiments an equivalent dose of the drug administered as a single i.p. injection was associated with loss of weight and with a decreased physical activity in 60% of the XPA deficient animals. Both WT and XPC-knockout mice showed under the same conditions neither the signs of constitutional toxicity nor morphological signs of renal damage. In order to visualize possible neuronal damage all obtained nervous tissues were examined morphologically (HE-staining). Prominent morphological changes in the tissue structure of DRG, including shrinkage of the cytoplasm and nuclear condensation in the presence of an intact plasma membrane, were found in both DNA repair deficient strains already 48 h following cisplatin administration. In particular DRG of $XPC^{-/-}$ mice were characterized by massive loss of neuronal cells. In contrast to these findings, the morphological integrity of DRG in WT mice was mostly good preserved both after low dose and high dose cisplatin treatment (figure 12). Thus, the enhanced formation and persistence of cisplatin adducts in the genomic DNA of NER deficient nervous cells had drastic morphological consequences leading to cell death.

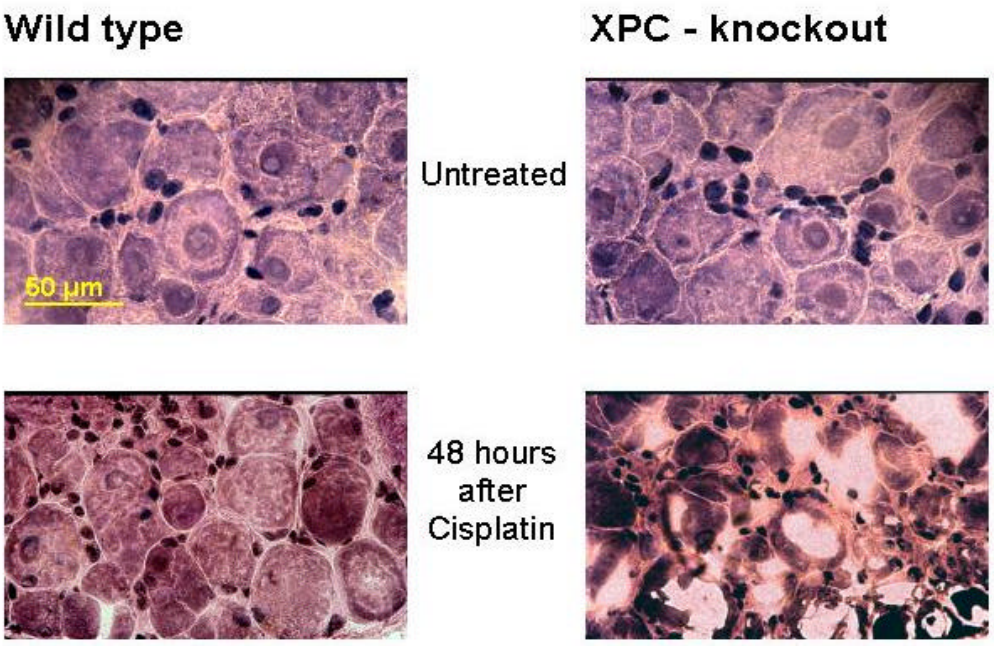


Figure 12 Extent of morphological damage in DRG tissue of wild type and DNA repair deficient mice after single treatment with 2 mg/kg cisplatin (HE-staining)

2.2.5 Functional evaluation of cisplatin-induced neurotoxicity in mice: electrophysiological examination of motor and sensory nerve fibers

In this study I have shown the enhanced formation of cisplatin-induced Pt-DNA adducts in DRG sensory neurons compared to the spinal cord cells. Furthermore, accumulation of DNA lesions in DRG neurons strongly correlated with the extent of morphological damage, increasing significantly in DNA repair deficient nervous cells. However, it still remains open to which extent accumulation and persistence of DNA lesions correlate with clinical signs of cisplatin-induced polyneuropathy. Therefore, a model was developed, in which both, DNA lesion quantification and electrophysiological studies could be assessed in the same animal.
In a pilot experiment I have investigated the correlation between the accumulation of Pt-DNA adducts in neuronal cells of WT and NER-deficient mice and functional impairment under chronic cisplatin treatment. WT and XPA-knockout mice were treated with cisplatin, 2 mg/kg, once a week or with sterile saline i.p. (controls).
Electrophysiological tests (M- and H-responses, motor [MNCV] and sensory nerve conduction velocity [SNCV] of the sciatic nerve) were performed at cumulative doses of 2, 4 and 8 mg/kg. The accumulation of cisplatin-induced DNA lesions in different neuronal cell types was analyzed in the ICA (4.8).
In cisplatin treated WT mice electrophysiological parameters did not differ from the control WT mice (table 1; figure 13).
In $XPA^{-/-}$ mice, starting at cumulative dose of cisplatin 4mg/kg, the amplitude of the H-reflex was reduced ($p<0.01$) and the H-reflex-related SNCV began to slow down (not significant). A cumulative dose of 8mg/kg caused a highly significant decrease in both sensory parameters compared to controls ($p<0.01$, table 1; figure 13). M-response and MNCV remained unaffected in both experimental groups (table 1; figure 13). No electrophysiological differences were observed between the NER-proficient and deficient controls (table 1).
The accumulation of Pt-DNA-adducts was dose dependent and approximately two-fold higher in DRG cells than in the same cell types of the spinal cord (table 2). The $XPA^{-/-}$ mice showed a significant increase in persisting DNA lesions compared to the WT counter points due to an impaired adduct removal in these cells (figure 14).
A strong association between the enhanced accumulation / persistence of Pt-DNA adducts in DRG cells and an early functional impairment of the sensory nerve fibers indicates that the amount of persisting DNA lesions is indeed very likely to determine the development of cisplatin-induced polyneuropathy.

Table 1 Electrophysiological parameters in WT and XPA$^{-/-}$ mice after different cumulative doses of cisplatin (chronic treatment, 2mg/kg per week) versus untreated littermates

	Group	Untreated Mean ± SEM	2mg/kg Mean ± SEM	4mg/kg Mean ± SEM	8mg/kg Mean ± SEM
MNCV m/s	**WT**	64.9 ± 5.5	61.5 ± 3.5	60 ± 2.5	57.2 ± 4.16
	P vs. control		NS	NS	NS
	XPA$^{-/-}$	66 ± 1.6	57 ± 2.6	57.4 ± 3.4	60 ± 10
	P vs. control		NS	NS	NS
M-response, mV	**WT**	5.3 ± 0.5	5.4 ± 0.2	5.2 ± 0.3	5.6 ± 0.4
	P vs. control		NS	NS	NS
	XPA$^{-/-}$	5.5 ± 0.39	5.9 ± 2.6	5.6 ± 0.4	4.6 ± 0.9
	P vs. control		NS	NS	NS
SNCV m/s	**WT**	43.5 ± 3.5	45.9 ± 1.2	48.3 ± 1.5	42 ± 0.7
	P vs. control		NS	NS	NS
	XPA$^{-/-}$	47.5 ± 3	47 ± 2.7	36.8 ± 3.1	32.25 ± 1
	P vs. control		NS	NS	< 0,01
H-reflex, mV	**WT**	1.51 ± 0.2	1.7 ± 0.1	1.95 ± 0.3	1.64 ± 0.3
	P vs. control		NS	NS	NS
	XPA$^{-/-}$	1.83 ± 0.2	1.7 ± 0.75	0.89 ± 0.1	0.8 ± 0.1
	P vs. control		NS	< 0.01	< 0.01

NS – not significant.

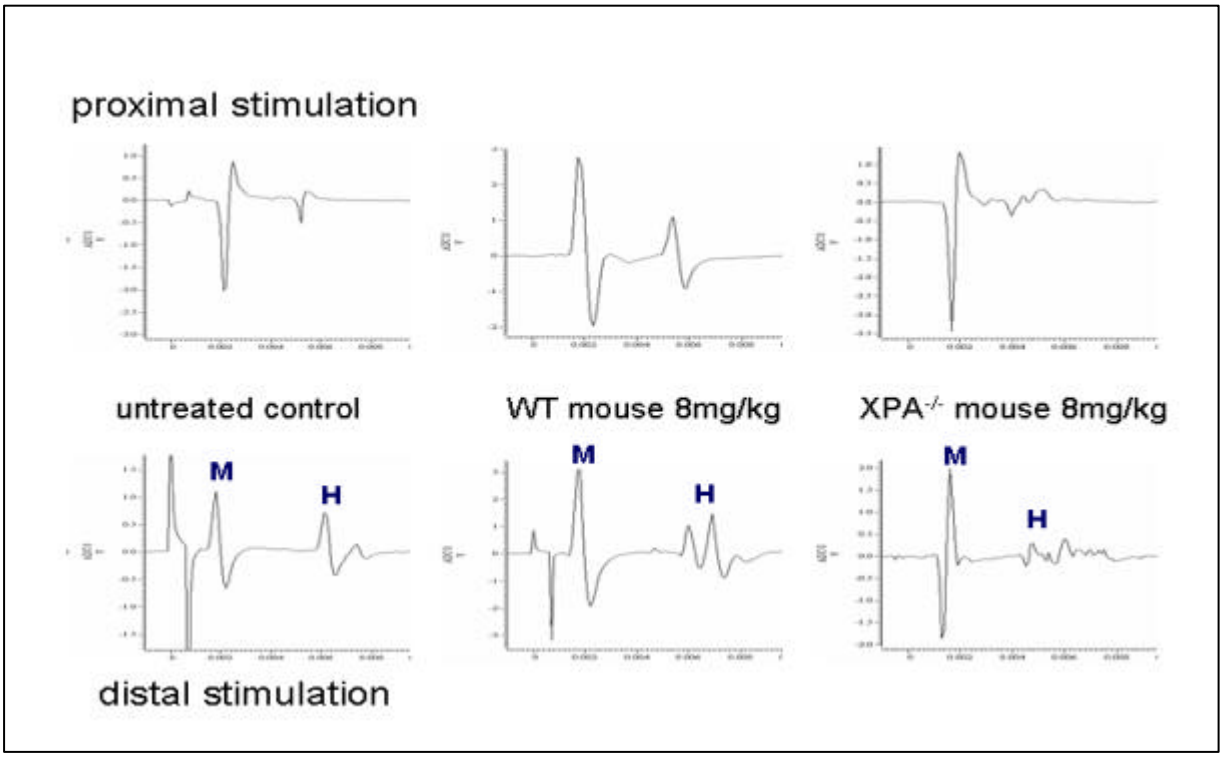


Figure 13 Electrophysiological examination of peripheral nerves as a clinical tool to demonstrate cisplatin-induced neuronal damage

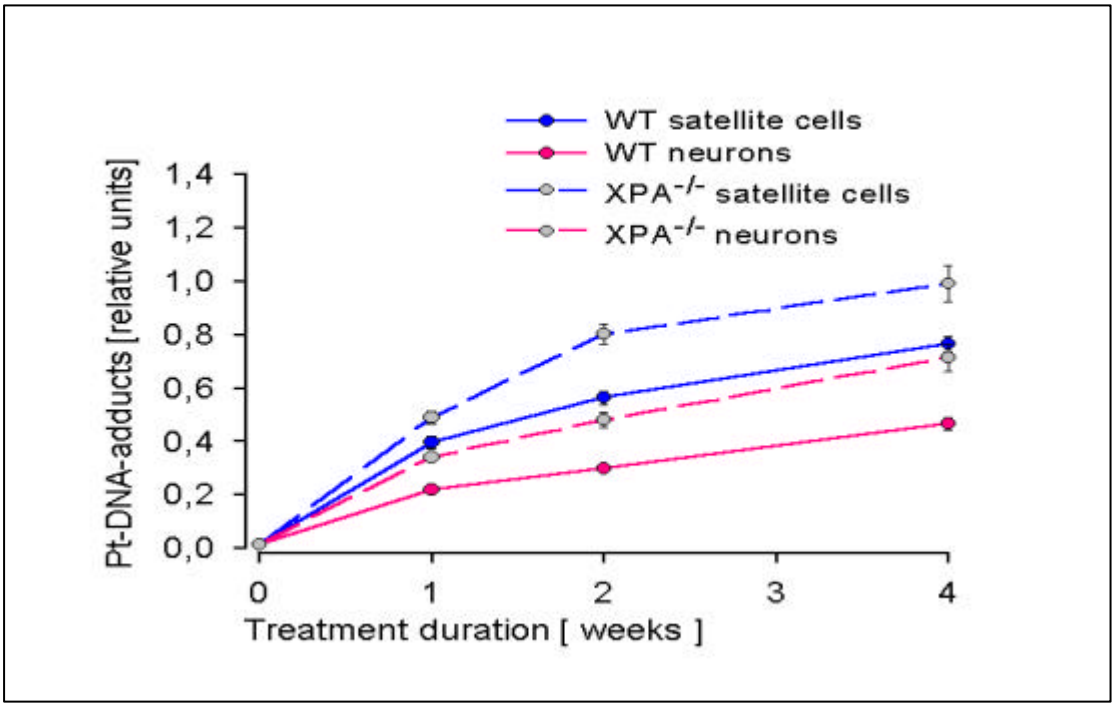


Figure 14 Accumulation of Pt-DNA lesions in DRG cells of WT and XPA$^{-/-}$ mice under chronic cisplatin treatment (2 mg/kg i.p. once a week)

Table 2 Accumulation of Pt-DNA adducts in the central and peripheral nervous system of WT and XPA$^{-/-}$ mice after different cumulative doses of cisplatin (chronic treatment, 2mg/kg once a week)

	Group	**2mg/kg** Mean ± SEM	**4mg/kg** Mean ± SEM	**8mg/kg** Mean ± SEM
DRG satellite cells	**WT**	0.40 ± 0.02	0.56 ± 0.02	0.77 ± 0.03
	P vs. control	< 0.05	< 0.01	< 0.01
	XPA	0.49 ± 0.02	0.80 ± 0.04	1.12 ± 0.07
	P vs. control	< 0.01	< 0.01	< 0.01
DRG neurons	**WT**	0.22 ± 0.01	0.29 ± 0.02	0.47 ± 0.02
	P vs. control	< 0.05	< 0.01	<0.01
	XPA	0.34 ± 0.01	0.48 ± 0.03	0.75 ± 0.04
	P vs. control	< 0.01	< 0.01	< 0.01
spinal cord glial cells	**WT**	0.19 ± 0.01	0.24 ± 0.02	0.30 ± 0.02
	P vs. control	< 0.05	< 0.01	< 0.01
	XPA	0.26 ± 0.01	0.32 ± 0.01	0.44 ± 0.04
	P vs. control	< 0.01	< 0.01	< 0.01
spinal cord neurons	**WT**	0.12 ± 0.01	0.15 ± 0.01	0.22 ± 0.01
	P vs. control	< 0.05	< 0.01	< 0.01
	XPA	0.21 ± 0.02	0.25 ± 0.01	0.31 ± 0.02
	P vs. control	< 0.01	< 0.01	< 0.01

3 DISCUSSION

3.1 Drug-induced peripheral sensory neuropathy is a severe and dose-limiting side effect of platinum based chemotherapy

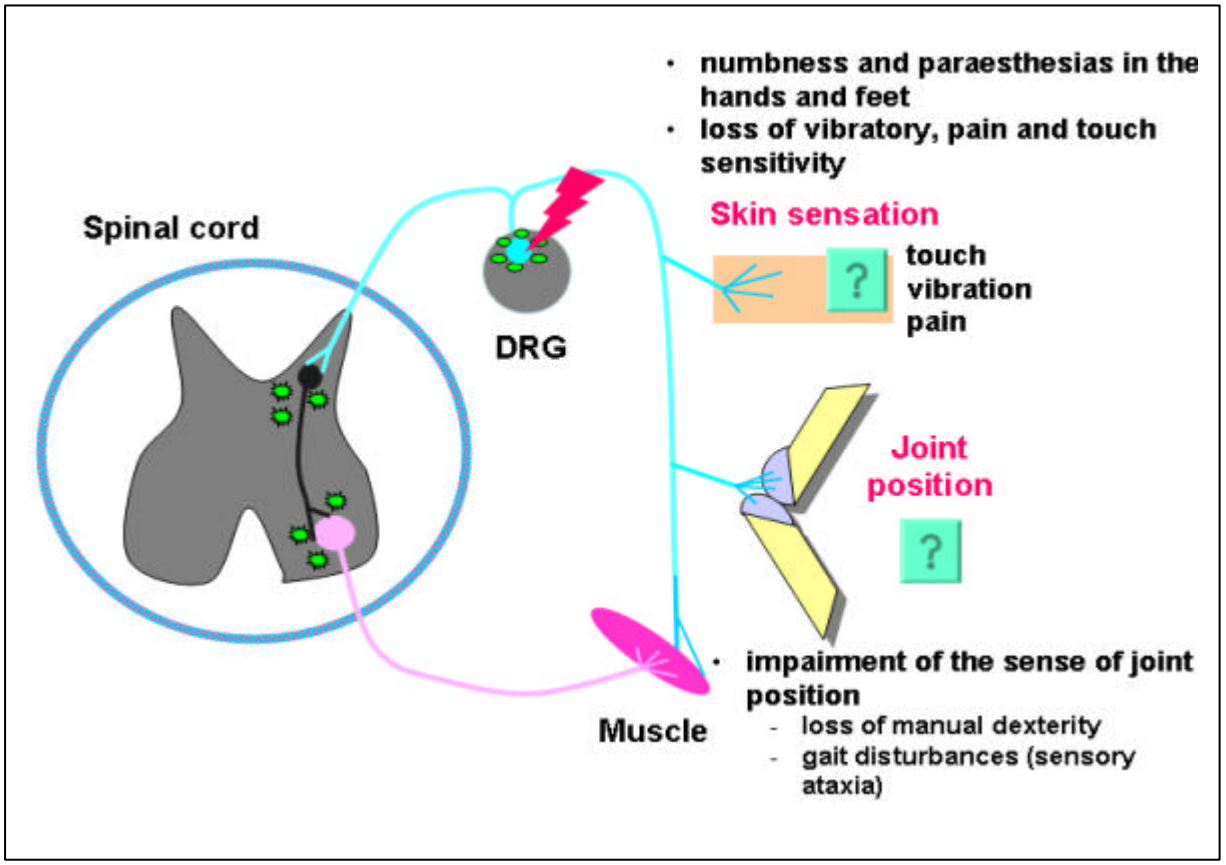


Figure 15 Cisplatin-induced neuropathy: clinical features

Cisplatin is one of the first line treatments for metastatic testicular and ovarian cancer. It is also used to treat bladder and small-cell lung carcinoma. Twenty percent of patients cannot be treated optimally because of peripheral sensory neuropathy – the major dose-limiting toxicity of cisplatin (1.3). Cisplatin-induced neurotoxicity is very often the reason for stopping the anti-tumor therapy or changing the dose regimen. Neurotoxicity depends on the cumulative dose and generally occurs in patients receiving more than 300 mg/m^2 (Grunberg et al., 1989; Walsh et al., 1982). Clinically it is characterized by a predominantly sensory neuropathy with diminished vibration perception, loss of tendon reflexes and discomforting paraesthesias starting in the lower extremities (Thompson et al., 1984; figure 15). In an advanced stage of the neuropathy the patient is ataxic with a pronounced gait disturbance due to impaired proprioception. Electroneurography detects a pure sensory axonal and secondary demyelinating neuropathy characterized by a reduction of sensory nerve action potential amplitude and slowing of sensory nerve conduction velocity (Ashraf et. al., 1990; LoMonaco et. al., 1992).

Symptoms of neuropathy may start even after cessation of therapy and can persist over the following months or years (Grunberg et al., 1989; Strumberg et al., 2002).
In most instances, cisplatin-induced neuropathy is only partly reversible and in up to 50% of cases damage is completely irreversible (Alberts & Noel, 1995; Strumberg, 2002). Therefore, it represents an important and persistent limitation of quality of life even when the tumor has been successfully treated by the drug. Other platinum compounds in active or experimental clinical use, including carboplatin and oxaliplatin, are also associated with sensory neurotoxicity to various degrees (Lévi et al., 1997; Machover et al., 1996; Siddiqui et al., 1997).
However, the underlying molecular mechanism of cisplatin-induced neurotoxicity remained obscure up to now. A rational driven approach to reduce neurotoxic side effects of cisplatin and probably of other DNA-reactive drugs is still to be developed.

3.2 Extent of DNA platination, activity of Nucleotide Excision Repair and tolerance to persisting Pt-DNA lesions determine the cell-type specific sensitivity to cisplatin

The chemotherapeutic mechanism of cisplatin is believed to involve intrastrand adducts formed during cisplatin-DNA interaction in cancer cells (Rosenberg, 1985). Cisplatin-induced bivalent Pt-DNA intrastrand adducts inhibit replication and transcription and can lead to the programmed cell death (apoptosis) of the target cells (1.4; Fichtinger-Schepman et al., 1995).
The degree of cisplatin-induced DNA damage as well as the resultant pathophysiological events are known to be cell type specific and interindividually variable. Different pharmacokinetic factors, such as kidney clearance, blood-brain barrier, drug import and export through the cellular membrane as well as cytoplasmatic detoxification, influence adduct formation (1.4.2). The adduct elimination depends on the activity of the nucleotide excision repair (NER) – a multistep DNA repair pathway, which includes the activity of the Xeroderma pigmentosum proteins XPA – XPG (1.4.3; El-Mahdy et al., 2000; Sancar, 1996; Smith et al., 2000; Wani et al., 1999). The capacity of this repair mechanism is rather inhomogeneous in different cell types (Kelland, 1994; Masuda et al., 1988). Finally, the individual degree of tolerance to persisting Pt-DNA lesions determines the cell survival or apoptosis (1.4.4). It has been demonstrated in an animal study that DNA adducts are formed in DRG neurons when exposed to cisplatin (Meijer et al., 1999). Further on, cisplatin was shown to induce apoptosis in DRG neurons *in vitro* and *in vivo* (Gill & Windebank, 1998) as well as in DRG satellite cells *in vivo* (Sugimoto et al., 2000). However, so far there was no *in vivo* evidence that the high sensitivity of DRG neurons and satellite cells to cisplatin might be due to the enhanced accumulation and / or persistence of Pt-DNA adducts in these cells. Therefore the aim of the present study was to investigate accumulation and repair of specific DNA adducts in different cell types of nervous tissue as critical parameters for the cisplatin-induced neurotoxicity.

3.3 The Immuno-Cytological Assay (ICA) is a suitable tool for the single cell quantification of specific Pt-DNA adducts in murine nervous tissues

Accumulation and repair of Pt-DNA adducts are the unambiguous determinants of cisplatin cytotoxicity and their prognostic significance definitely outmatches the value of other measurable parameters such as plasma platinum concentration, expression of certain pro- or antiapoptotic factors or nucleolar size (McKeage et al., 2001; Schellens et al., 1996; van de Vaart et al., 2000).

Until now, limited tools to quantify and monitor structural changes in DNA following the administration of DNA-reactive drugs inhibited efforts to gain further insights into the interdependence of molecular and morphological changes in cells of the central and peripheral nervous system. The development of antisera against platinated DNA opened the way for the detection of low-levels of Pt-DNA adducts *in vivo*. Immunocyto- and histochemical application of suitable antibodies allows their morphological localization in both tumor and normal tissues. However, the previous attempts to localize and quantify cisplatin-induced Pt-DNA lesions in DRG tissue, using immunohistochemical techniques, resulted in controversial findings: according to Terheggen et al. (1989) the level of Pt-DNA binding in DRG satellite cells equaled that in liver cells, but DNA platination could not be shown in DRG neurons, whereas Meijer et al. (1999) could observe Pt-DNA adduct formation in DRG neurons in 43% of cisplatin treated rats, but only occasionally in DRG satellite cells. This discrepancy clearly demonstrates that much more precise immunostaining techniques are required to realize molecular dosimetry of these lesions.

The quantitative *in vivo* analysis of cisplatin-induced DNA lesions in different cell types of central and peripheral nervous system has been successfully realized in the present study at the single cell basis. This goal has been achieved using the combination of a newly developed monoclonal antibody against cisplatin-induced DNA adducts (anti-Pt-(G-G) MAB) with digital immune- and DNA-fluorescence microscopy in the immuno-cytological assay. It allows for the first time the cell type identification and the measurement of nuclear DNA platination levels in nervous cells within the same specimen (2.1; figure 6). Further advantages of this approach are the high specifics and sensitivity of adduct detection due to the application of anti-Pt-(GG) MABs and secondary sandwich immunostaining. This proved to be especially important for the quantitative adduct measurement in a broad range of nervous tissues, characterized by relatively low amounts of Pt-DNA adducts.

3.4 Quantification and monitoring of Pt-DNA adducts in different neuronal and glial cell types

Accumulation of specific Pt-DNA adducts was observed in all investigated cell types of the central and peripheral nervous system following the single i.p. administration of cisplatin, regardless of the dose intensity (2 or 10 mg/kg) and the DNA repair proficiency status.

The quantification of Pt-DNA adducts revealed different accumulation pattern in the investigated cell types.

First, DNA damage was more pronounced in DRG cells (outside the blood-brain barrier) than in the same cell types of the spinal cord: under therapeutic-dose treatment DRG neurons accumulated about 1,5 times higher adduct amounts than the spinal cord neurons and DRG satellite cells – 1,75 times higher than the glial cells of the spinal cord (see 2.2.2; figure 7). This finding is in good agreement with many previous reports on pharmacokinetics and tissue distribution of cisplatin itself or its DNA reaction products (Boven et al., 1985; Johnsson et al., 1995; Lange et al., 1973; Litterst et al., 1976 & 1979; Roelofs et al., 1984; Stewart at al., 1982). Although adduct levels within the CNS, especially in glial cells, increased with escalating cisplatin dose, the DRG cells remained nevertheless most affected. Hence, the reason for the predominant peripheral sensory neuron toxicity may be the drug access rather than the selective neuronal vulnerability. Cisplatin cannot easily cross the blood-brain barrier (BBB) and, therefore, motor neurons and other central nervous system cells are not directly exposed to toxic levels of the drug. However, if the protection by the BBB is weakened, e.g. in the cases of brain tumors or by high dose mannitol coadministration, central neurotoxicity can also be observed (Fountzilas et al., 1991). Similarly, high levels of Pt-DNA adducts were reported in autopsy brain samples from patients, who received multiple cisplatin treatments amounting to high cumulative doses (Poirier et al., 1992).

Second, there was an obvious cell type specific accumulation of adducts within the nervous tissues: glial and satellite cells accumulated more DNA lesions than neurons of the same anatomical structures. In particular, DRG satellite cells showed 1.4-fold higher extent of DNA platination than the corresponding neurons. The prominent Pt-DNA adduct accumulation in dorsal root satellite cells has been described elsewhere (Terheggen et al., 1989). This finding is also in agreement with several previous reports, where the satellite cells were shown to develop severe ultrastructural changes after cisplatin exposure (Cece et al., 1995; Corsetti et al., 2000; Sugimoto et al., 2001). One explanation might be that the dorsal root neurons are ensheathed by a capsule of satellite cells, which is usually directly apposed to the neuronal cell bodies. Therefore, DRG neurons are completely separated from the surrounding environment and can only contact the extracellular space through the narrow clefts between the wrappings of satellite cells (Tennyson & Gershon, 1993; Pannese, 1981). Satellite cells, on the contrary, are facing extracellular space and are directly exposed to the tissue cisplatin concentrations. Similar relationships regarding the mechanical support and the trophic function are known for neuronal and glial cells within the CNS as well.

Interestingly, a very low adduct formation has been observed in Schwann cells of the sciatic nerve throughout all experiments.

The Pt-DNA adduct levels in these cells under the therapeutic dose of cisplatin were about 3.7 times lower than in the DRG satellite cells and even lower than in the spinal cord cells. Likewise surprisingly low adduct levels have been previously described in the peripheral blood lymphocytes, which are directly exposed to the serum cisplatin concentrations (Liedert, 2001; Terheggen et al, 1988; van de Vaart et al., 2000), as well as in testis, where the Pt-DNA adducts were restricted to interstitial cells and absent in germ cells, although testicular tumors are known to be extremely sensitive to cisplatin (Johnsson et al., 1995; Terheggen et al., 1987). Such phenomena reflect the plurality and complexity of pharmacokinetic factors, which determine the adduct formation within the particular cell type. As recently shown, low adduct levels in specific cell types can be due to reduced influx, accelerated export or augmented metabolic inactivation of cisplatin (Giaccone, 2000; Liedert et al., 2003). A relatively low DNA platination in Schwann cells within the peripheral nerve might be due to the protecting role of the endo- / perineurium, serving as a “blood-nerve barrier” (Low, 1976; Tennyson & Gershon, 1993), as well as to a relatively poor vascularisation, compared to DRG, which is supplied by fenestrated capillaries (Gill & Windebank, 1998; Lieberman, 1976). However, the molecular mechanisms responsible for such different adduct formation rates in satellite and Schwann cells remain obscure.

The organ-specific pharmacokinetics of cisplatin has been described in several previous reports (Boven et al., 1985; Fichtinger-Schepman et al., 1989; Johnsson et al., 1995; Litterst et al., 1979; Terheggen et al., 1987). The present study has performed for the first time a monitoring of DNA platination at the level of individual cells within the CNS, DRG and the peripheral nerves.

The time profiles and the elimination parameters of Pt-DNA-adducts measured in different cell types of nervous tissue were similar and correlated with those recorded for kidney and liver tissue (Liedert, 2001) with some exceptions. This suggests that the DNA repair system responsible for the removal of Pt-intrastrand crosslinks is equally active in the investigated cells. Peak adduct levels were reached at 24h after treatment (figure 9). Thus, the formation of adducts seems to be rate limiting compared to the tissue distribution of the drug: peak tissue platinum concentrations are normally achieved at 15 min after i.p. administration (Johnsson et al., 1995; Fichtinger-Schepman et al., 1989). This time-lag could be due to the relatively slow transformation of intermediate monovalent platination products into bivalent adducts, as detected by ICA, or because of the partial removal of adducts from nuclear DNA by NER mechanism already at the intermediate stage (Liedert, 2001).

The biphasic elimination of DNA lesions included an intensive repair phase, when up to 60% of lesions were removed within the next 24h – 6 days, depending on the amount of the initial adduct burden, and a delayed terminal elimination phase characterized by slowly decreasing plateau of unrepaired adducts. The fast initial decline in adduct levels could reflect to a certain extent that cells with high initial adduct burden died off. Interestingly, about 30% of the initially formed Pt-DNA adducts are obviously “repair-resistant” and can be detected in DRG cells even two weeks after cisplatin administration (figure 9).

Hence, both, the neuronal and glial cells seem not be capable to completely remove all Pt-(GG)-intrastrand crosslinks from the genome, even in the DNA repair proficient WT mice. This finding might be important for the clinical situation, when the critical amount of unrepaired adducts could pile up during repeated “chronic” exposure to the drug.

Taken together, the highest adduct levels were found in the dorsal root neurons and in the supporting satellite cells, which proved to be more affected. The satellite cell damage and especially the disorganization of their sheath, which serves as the only protective barrier for the DRG neurons, may facilitate the neuronal damage and therefore largely contribute to the development of cisplatin-induced polyneuropathy.

What is the clinical relevance of these results? The pharmacokinetics of chemotherapeutic agents, including cisplatin, are similar in humans and in mice (Kubota et al., 1993). Further on, the dosages used in both low-dose and high-dose treatment schedules were chosen in order to represent the most frequently used therapeutic dose (50 mg/m^2) and the cumulative dose (300 mg/m^2), at which the first clinical signs of neuropathy occur in most of cisplatin treated patients. This suggests that the presented here mouse model may provide a fair estimate of the formation and repair of cisplatin-induced DNA lesions in human nervous system.

3.5 Deficiency in XPA or XPC activity leads to increased adduct accumulation in neuronal cells and enhanced acute cisplatin toxicity

The XP (Xeroderma pigmentosum) – gene family encodes a number of essential components of the nucleotide excision repair (NER) pathway, which itself is a part of the comprehensive DNA damage response network of mammalian cells. Mutations and polymorphisms in XP-genes are associated with UV-hypersensitivity, cancer predisposition and premature aging (De Boer, 2002; van Steeg et al., 2000).

The present study has addressed the question to which extent the NER pathway and its components are involved in the repair of Pt-DNA lesions within the nervous system. Mice deficient in XPA or XPC gene functions were analyzed for the repair kinetics of Pt-(GG) crosslinks in the nuclear DNA of neuronal and glial cells of the DRG, spinal cord and peripheral nerve.

As already observed in wild type animals, the adduct formation peaked 24 hours following cisplatin administration and the highest adduct levels were found in DRG cells (2.2.3; figure 11). However, XP-knockout animals were characterized by two-fold higher peak adduct levels compared to WT mice both, in the cells inside and outside the BBB. Interestingly, while the XPA$^{-/-}$ mice were still capable of limited Pt-DNA adduct removal, in XPC$^{-/-}$ mice these lesions were not repaired at all and persisted at the peak level for more than 5 days.

Thus, this newly established *in vivo* model is perfectly suitable to analyze the consequences of different adduct formation / persistence due to the different DNA repair capacity in primary cells on a homogeneous genetic background.

The next question addressed was whether this increase in adduct accumulation is indeed followed by clinically relevant morphological and functional changes. A morphological examination (HE-staining) revealed prominent structural changes in DRG tissues of both knockout strains and massive loss of $XPC^{-/-}$ neurons already after a single cisplatin treatment. One thus could find a strong correlation between the enhanced adduct accumulation / long term persistence of DNA lesions in DRG neurons and the extent of morphological damage following cisplatin exposure (2.2.4; figure 12).

The results of the pilot electrophysiological experiment proved to be nonetheless demonstrative. WT and XPA-knockout mice were administered cisplatin chronically for several weeks and the correlation has been investigated between the accumulation of Pt-DNA-adducts in neuronal cells and functional impairment in peripheral nerves (2.2.5). An insufficient NER function in the $XPA^{-/-}$ DRG neurons resulted in a high accumulation of unrepaired DNA lesions within these cells. To achieve an adduct level comparable to that in $XPA^{-/-}$ mice at a cumulative dose of 4 mg/kg cisplatin, WT mice had to receive a two-fold higher cumulative dose. Furthermore, the enhanced persistence of Pt-DNA adducts in DRG cells of NER deficient $XPA^{-/-}$ mice was strongly associated with an early functional impairment of sensory nerves: both, the amplitude of the H-reflex and the SNCV decreased significantly ($p < 0.01$) after the cumulative dose of 8 mg/kg cisplatin, whereas NER competent WT mice, which DRG cells were bearing 35% less platinum-DNA lesions, remained electrophysiologically intact under the same cumulative dose.

Thus it seems plausible that the amount of persisting DNA lesions in DRG neurons might be the critical parameter which determines the development of cisplatin-induced polyneuropathy. The current study conducted by our group is aimed to prove, whether in WT mice at higher cumulative doses of cisplatin the amount of unrepaired DNA lesions in DRG cells would correlate with the severity of functional changes, according to the dose-dependent character of sensory polyneuropathy known for patients under the long-term cisplatin treatment.

3.6 Role of XPA and XPC proteins on the repair processing of Pt-DNA adducts in the target cells of nervous system

The clearly different character of Pt-DNA adduct repair kinetics observed in the two investigated NER deficient strains reflects different function of both XP proteins in the recognition and repair of DNA lesions which can be described by the following model (figure 16).

Cisplatin interacts with the DNA in two steps: first intermediate monovalent platination products are formed and second, if not removed within the next several hours, they are transformed into “late” bivalent adducts (GG-crosslinks) which are responsible for the drug cytotoxicity. According to our data it is most likely that the augmented formation rate of GG-crosslinks in XPA- and XPC-deficient cells is due to their inability to remove the platination products from DNA already at the intermediate mono-adduct stage.

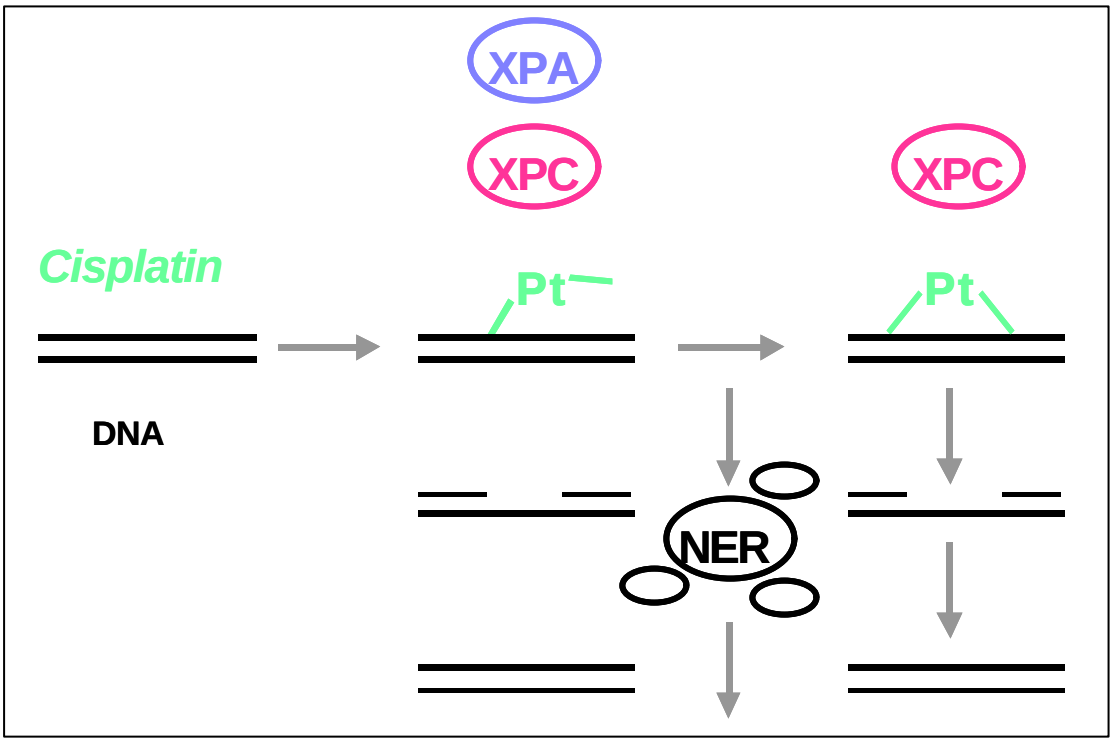


Figure 16 Model for the substrate specificity of XPA and XPC proteins towards mono- and bivalent Pt-DNA adducts

To say it in other words, the damage recognition and initiation of the repair process must happen in NER-proficient WT cells prior to the relatively slow crosslinking reaction resulting in comparatively low Pt-(GG) levels in these cells. This “early” repair seems to be under the control of both, XPA and XPC proteins. Once formed the crosslinks are recognized and excised by the NER mechanism only in the presence of a functional XPC protein, whereas XPA is not completely essential for this process. This observation is in good agreement with the results reported by Zhen et al. (1993). In accordance with our data, this study on the gene-specific repair demonstrated that the human fibroblasts from XP patients lacking active XPA protein still possessed a limited nucleotide excision repair capacity and were able to remove approx. 22% of cisplatin-induced DNA crosslinks.

Thus, in our mouse model the impairment of DNA lesion recognition appears to be the most deleterious mechanism to initiate the cascade of neuronal cell damage and cell death under cisplatin treatment. With regard to the clinical situation it might become a critical pre-dispositional factor for the onset and / or severity of cisplatin-induced polyneuropathy since a broad interindividual variability in DNA repair capacity was observed in humans (Buschfort et al., 1997; Buschfort-Papewalis et al., 2002).

3.7 New insight into the pathophysiology of cisplatin-induced polyneuropathy

Predominant DNA-damage in DRG neurons and satellite cells may reflect a crucial mechanism of sensory polyneuropathy following cisplatin exposure.

First of all, this finding is in good agreement with previous animal investigations and autopsy studies on cisplatin-treated patients. Platinum levels in post mortem tissues are greatest in the dorsal root ganglia, although it does accumulate in peripheral nerves, ventral roots, and dorsal roots. Levels in the central nervous system are dramatically lower, indicative of protection by the blood-brain barrier (Gregg et al., 1992; Poirier et al., 1992 & 1993; Roelofs et al., 1984; Stewart at al., 1982; Terheggen et al., 1989; Thompson et al., 1984;).

Potential anatomic sites of the toxic effect include the DRG neuron cell body, the DRG axon, and supporting cells within ganglia and peripheral nerves. While axonal degeneration has been observed in post mortem studies, this appears to be a late-stage event secondary to changes in the cell body (Roelofs et al., 1984). Additionally, no evidence was found of primary demyelination (Thompson et al., 1984). This fact excludes Schwann cells at least from the primary targets of cisplatin neurotoxicity and my own observation of low Pt-DNA adduct levels in Schwann cells supports this idea.

The impairment of fast axonal transport due to the microtubuli cross-linking caused by platinum-protein interaction was discussed as a potential mechanism contributing to the cisplatin-induced neurotoxicity (Boekelheide et al., 1992; Gao et al., 1995). However, no changes in microtubuli morphology have been observed under cisplatin exposure (Russell et al., 1995). The data on whether cisplatin affects the function of ion channels are scant.

Cisplatin does not inhibit Na-K ATPase activity in renal tissue *in vivo* (Uozumi & Litterst, 1985) and shows no significant influence on excitability parameters of isolated peripheral nerves (Grosskreutz et al., 2000; Quasthoff & Hartung, 2002).

Furthermore, fast axonal transport shows uniformity of velocities across species in motor and sensory nerves, in myelinated fibers of varying sizes (Ochs, 1972) and in unmyelinated fibers (Ochs & Jersild, 1974). Since cisplatin selectively affects the function of sensory nerve fibers (predominantly large myelinated), the primary impairment of fast axonal transport seems unlikely to be the underlying mechanism of cisplatin-induced neurotoxicity.

The data of my experiments now provide evidence, that Pt-DNA crosslinks are indeed being formed in the nervous cells under *in vivo* cisplatin exposure and that the predominant DNA damage occurs in the DRG neurons and satellite cells (2.2).

DNA damage is an initiator of programmed cell death and this is one of the mechanisms by which DNA-reactive drugs exert their antitumor potential. Cisplatin has been shown to induce apoptosis in DRG neurons *in vitro* and *in vivo* (Fischer et al., 2001; Gill & Windebank, 1998) as well as in the trigeminal ganglion satellite and Schwann cells *in vivo* (Sugimoto et al., 2000). Hence, it seems quite plausible, that the molecular basis for both the cytotoxic action and the neurotoxic action of cisplatin is identical.

Furthermore, the enhanced accumulation / persistence of Pt-DNA adducts in DRG cells was strongly associated with morphological signs of neurotoxicity as well as with an early functional impairment of peripheral nerves as shown in the present *in vivo* study using neurophysiological and histological techniques.
Thus, one can conclude that the amount of persisting DNA lesions might determine the development of cisplatin-induced polyneuropathy.

3.8 Mechanisms of cell death following cisplatin administration

The extent of morphological changes after cisplatin, including shrinkage of the cytoplasm, nuclear condensation and massive loss of neuronal cells, increased with both enhanced formation and persistence of DNA lesions, as observed in DRGs of $XPA^{-/-}$ and $XPC^{-/-}$ mice (2.2.4; figure 12). The precise mechanisms of cell death following cisplatin exposure, however, are still under debate. As DRG cells usually don't proliferate the interference of Pt-DNA adducts with replication processes and the typical cell cycle arrest in G_2 after cisplatin are clearly not the triggering events of apoptosis in this tissue. Hence, the block of transcription is more likely to play the crucial role. This view is supported by the hypersensitivity to cisplatin of mice lacking a functional XPA as this protein is involved in both, global genomic and transcription coupled repair mechanisms (Furuta et al., 2002). Moreover, McDonald & Windebank (2002) have recently shown in an elegant mouse DRG explantation model, that cisplatin-induced cell death of neurons is associated with the activation of the mitochondrial stress pathway via translocation of the proapoptotic Bcl2-associated X protein (BAX) to the mitochondrial membrane followed by cytochrome c release. This observation together with the finding that the triggering of this cascade is independent from functional fas or caspase-8 activity indicates that the mitochondrial pathway is the major signaling pathway to apoptosis in cisplatin-exposed neurons. Interestingly, bax translocation to mitochondria in this model and the onset of cell death could be reduced significantly by co-application of high dosed submaxillary gland nerve growth factor (NGF). Whether NGF interferes with the persistence of specific lesions in DNA (e.g. by stimulating related repair mechanisms) or abrogates the signaling to bax translocation remains to be tested.

3.9 Outlook: from understanding the molecular basis of cisplatin-induced neurotoxicity to the development and testing of neuroprotective agents

Cisplatin-induced neurotoxicity is first of all a clinical problem. Understanding its pathophysiology is in the focus of basic research. Although there are still many questions to be answered, this particular study has succeeded to develop a mouse model, which enables new insights into molecular mechanisms of cisplatin-induced polyneuropathy. According to my experimental data, it is very plausible that cisplatin-induced DNA lesions represent the primary molecular basis of neurotoxicity. The amounts of persisting Pt-DNA adducts in the neuronal target cells (DRG neurons) together with the activity of DNA repair mechanisms determine the extent of morphological damage and neurological deficit following cisplatin exposure. The adduct formation is influenced by a number of different pharmacokinetic factors and is cell type specific (Johnsson et al., 1995; Liedert, 2001; Mustonen et al., 1989; Terheggen et al., 1987; own data). Thus, it seems quite reasonable to consider the platinum-DNA interaction as a therapeutic target: chemoprotective agents should either selectively hamper adduct formation or should prevent adduct-induced cellular damage in nervous cells without influencing an antineoplastic efficacy of cisplatin. Potential chemoprotective substances could be tested in the present mouse model according to these requirements. Nerve growth factor, involved in functional maintenance of mature DRG sensory neurons and antagonizing the effects of neurotoxic damage to these cells (Donnerer, 2003; Lindsay, 1992; Kirstein & Farinas, 2002; Windebank & McDonald, 2002), has recently been shown to rescue DRG cells *ex vivo* from cisplatin-induced apoptosis (McDonald & Windebank, 2002). Hence, this substance could become under consideration as a candidate for such neuroprotective drugs.

Another key factor of cisplatin-induced neurotoxicity – the DNA repair capacity – seems to be rather a genotype specific feature of highly differentiated cells, which does not depend on the metabolic peculiarities of the drug-cell interaction within some tissue. Although, DNA repair can obviously not be positively influenced by pharmacological interventions recently available, the further investigation of its genuine mechanisms and especially of its key regulatory genes is of great importance. A substantial interindividual heterogenity in the DNA repair capacity has been observed in the human population (Buschfort-Papewalis et al., 2002). A low expression of genes, which functions are crucial for the repair processing of Pt-DNA adducts, can, therefore, be regarded as a serious risk factor for the development of polyneuropathy under cisplatin treatment.

The method developed in this work allows a precise analysis and monitoring of platinum-DNA lesions in the structures of the central and peripheral nervous system *in vivo*. Thus its possible field of application includes targeting both critical points of cisplatin-induced neurotoxicity: designing new neuroprotective drugs and gaining further insights into the mechanism of DNA repair.

4 MATERIALS AND METHODS

4.1 Animals

C57Bl/6 mice were used as wild type mice (WT) throughout all experiments. XPA- and XPC-knockout mice were used as NER deficient animals.
XPC-deficient mice (Berg et al., 1998; Cheo et al., 1997) were kindly provided by Dr. L. Mullenders (University Leiden, NL) with their knockout status verified.
XPA-deficient mice (Nakane et al., 1995) were a generous gift from Dr. K. Tanaka (University of Osaka, Japan).
WT and $XPA^{-/-}$ mice were propagated by house breeding in the Institute of Cell Biology, University of Essen, Germany. Homozygocity of $XPA^{-/-}$ mice was verified by PCR analysis (4.2).
Mice were housed under the diurnal lighting conditions, adhered to a dark phase between 18.00 – 6.00, allowed daily food "Zuchthaltungsfutter Maus-Ratte 10 H 10" (Eggersmann) and water ad libitum, and additionally oat bran once a week. Every half a year two animals from each strain were checked for pathogenic microorganisms.
All experiments have been approved by the state animal welfare board.

4.2 Genotype verification of XPA-knockout mice

To verify the XPA-knockout status of mice a 0,4 – 1,2 cm long piece of tail tip was taken from each 4 weeks-aged animal under the local anesthesia. The DNA was isolated with "Qiagen DNeasy Kit" (4.2.1), quantified with "SYBR Green I" test (4.2.2) and assessed in PCR (4.2.3).

4.2.1 DNA isolation

The high-molecular DNA isolation from the mouse tail tissue was performed with "DNeasy Kit" (Qiagen) according to the instruction of the manufacturer.
The purity of the prepared nucleic acids has been tested photometrically at 260 nm and 280 nm (Spectrophotometer: model 150-20, Hitachi) according to Sambrook et al., 1989. The E_{260}/E_{280} ratio of 1.8 – 2.0 was judged as a neat DNA preparation. The DNA integrity was verified with agarose (0.9%) gel electrophoresis (4.2.4).

4.2.2 DNA quantification

The DNA concentration was quantified with the "SYBR Green I" test (Molecular Probes).
The required standards with concentration range of 0.1 – 1 µg/ml were prepared in TE buffer from high-molecular weight DNA (Roche) and adjusted UV-photometrically (Spectrophotometer model 150-20, Hitachi) at 260 nm (50 µg double strand DNA / ml corresponds to 1 A_{260}).
Each well of 96-well-plate (Maxisorp, Nunc) was filled with either 50 µl of the standard DNA or 50 µl of the DNA probe to be analyzed.

Each standard dilution and each DNA sample were applied twice. 50 µl of the DNA reactive fluorescent dye “SYBR Green I” diluted 1:10.000 in TE were added into each well. The test plate was then incubated protected from light at 25°C for 15 min. The measurement was performed with the excitation at 485 nm and the emission of DNA-fluorescence detected at 530 nm in a fluorescence plate reader (FL 500, PC-program FL 500 1.D.1, Bio-Tek Instruments). The detection limit was determined as 0.001 µg/ml (50 pg DNA/well).

4.2.3 PCR technique

The PCR was performed according to the modified protocol of Nakane et al., 1995, with the final mixture volume of 50 µl.
The following primers were applied:

Primer A (Upper primer: wild type)
5´ GTG GGT GCT GGG CTG TCT AA 3´
M_r: 6.300; 5.30 nmol/A_{260}

Primer B (Lower primer: wild type / knockout)
5´ATG GCG TGG GTT CTT CTT C 3´
M_r: 6.210; 5.44 nmol/A_{260}

Primer C (Upper primer: knockout):
5´ ATG GCC GCT TTT CTG GAT TC 3´
M_r: 6.170; 5.62 nmol/A_{260}

The PCR reaction mixture (40 µl) was prepared under contamination control according to the following scheme and kept on ice until adding the DNA.

Component	Concentration of the original solution	Entry volume [µl]	Concentration in the mixture
H_2O	--	34	--
Primer A	400 µM	0.03	250 nM
Primer B	400 µM	0.03	250 nM
Primer C	400 µM	0.03	250 nM
d NTP (Amersham Pharmacia)	25 mM	0.4	200 µM
AccuTherm – Buffer	10 x	5	1 x
AccuTherm-Polymerase (GeneCraft)	5 U / µl	0.3	0. 03 U / µl

10 µl of the isolated DNA (20 µg/ml in H_2O) were added to the reaction mixture. The PCR was then performed under the following temperature profile (PCR-engine: MJ Research PTC 200, Biozym):

time	temperature	cycles
1 min	94 ° C	1
25 s	94 ° C	34
30 s	55 ° C	34
60 s	72 ° C	34
4 min	72 ° C	1

The PCR products were analyzed by electrophoresis in 3% agarose gel (4.2.4)

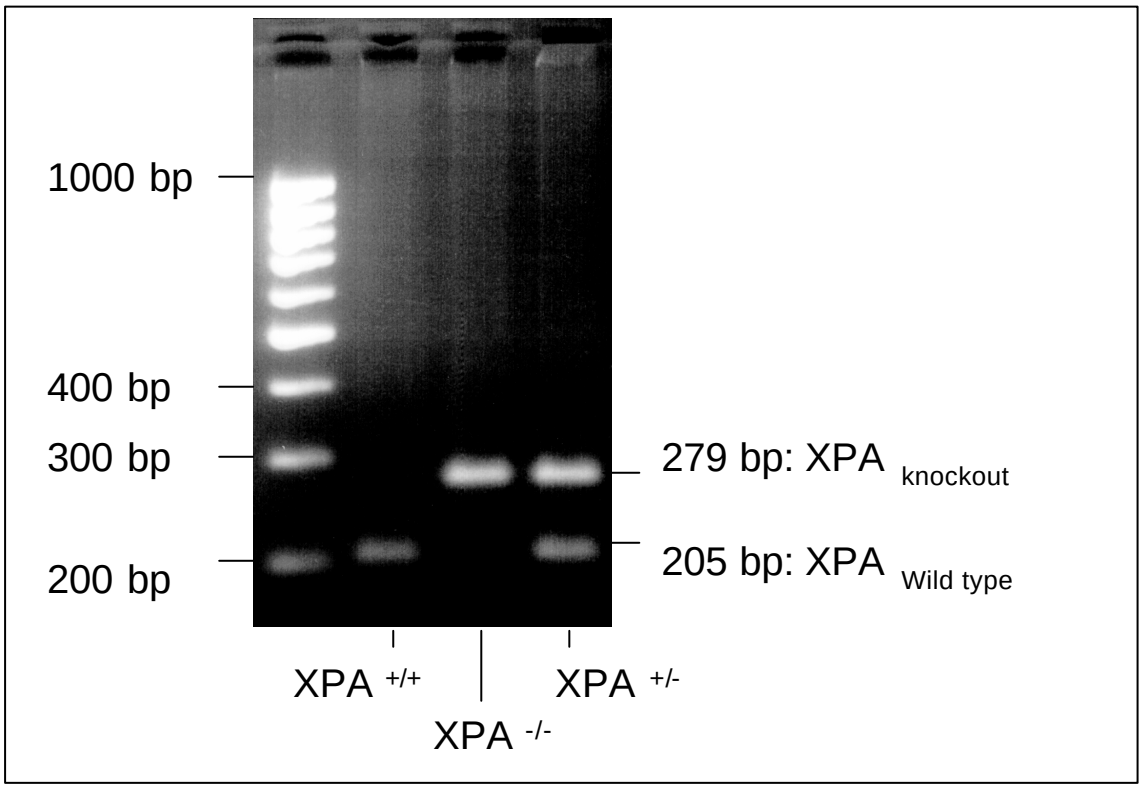


Figure 17 Genotype determination of hetero- and homozygous XPA-knockout mice (3% agarose gel)

4.2.4 Agarose gel electrophoresis

To verify the DNA integrity after the isolation from the mouse tail tip (4.2.1) and to analyze the products of PCR the following concentrations of agarose gel were used:

agarose concentration [%]	aim of application	mg of agarose for 50 ml TAE-buffer
0.9	DNA isolation (4.2.1)	450
3	PCR products (4.2.3)	1.500

The required amount of “Biozym Agarose” (0.9%) or “Biozym Small DNA Agarose” (3%) was solved in 50 ml of TAE-buffer and microwaved at 700 W for 2 min. 4 µl of ethidiumbromide were added into the gel before allowing it to solidify. 12.5 µl of each sample were supplied with 2.5 µl of 6x loading buffer and applied for electrophoresis which was performed in 1xTAE at 50 V for 2 hours. The “GeneRuler” 50 bp, 100 bp or 1kb DNA ladders (MBI) were used as standards. The results were evaluated with the UV-transilluminator (Bachmann).

4.3 Experimental design

Mice of experimental groups were treated with cisplatin (Platinex, Bristol Arzneimittel GmbH, München); control animals were treated with sterile saline. Injections were performed intraperitoneal (i.p.). Mice were weight and checked for signs of drug toxicity at least twice per week. Animals showing signs of distress were immediately and painlessly sacrificed.

To investigate cell type specific Pt-DNA adduct accumulation and repair in different cell types of nervous tissue, WT mice were treated with a single cisplatin dose of 2 or 10 mg/kg; $XPA^{-/-}$ and $XPC^{-/-}$animals received a single cisplatin injection of 2 mg/kg. Following cisplatin or sterile saline administration 6h, 12h, 1, 2 or 5 days after low dose treatment or 6h, 12h, 1, 3, 7 or 14 days after high dose treatment, animals were sacrificed by supramaximal anesthesia with diethyl ether.

To investigate the correlation between the accumulation of Pt-DNA-adducts in neuronal cells and functional impairment WT and XPA-knockout mice were treated with cisplatin, 2 mg/kg, once a week or with sterile saline i.p. (controls). To prevent renal damage, 1ml of sterile saline was injected i.p. immediately after each cisplatin treatment. Electrophysiological tests (M- and H-responses, motor [MNCV] and sensory nerve conduction velocity [SNCV] of the sciatic nerve, 4.9) were performed at cumulative doses of 2, 4 or 8 mg/kg one week after the last treatment. Mice were sacrificed immediately after the examination. Frozen tissue sections were prepared from DRG and spinal cord. The accumulation of cisplatin-induced DNA lesions in different neuronal cell types was analyzed in the ICA (4.8).

4.4 Preparation of tissue samples

The sacrificed animals were immediately and carefully perfused with 20 ml of sterile saline via intracardiac catheterization and underwent dissection. To prevent the nervous structures from early enzyme-induced postmortal morphological damage, the carcass was kept on ice during the organ dissection and the exposed organs were constantly rinsed with PBS (0.01M, pH 7.4) precooled to 4°C.

A midline dorsal longitudinal incision was made over the toracal and lumbar spine till sacrum. The lamina was gently exposed between the spinal muscles and laminectomy Th12-S1 was performed microscopically (stereomicroscope Stemi SV 6, lens W – Pl 10x / 1, Zeiss). The lumbar part of the spinal cord and the lumbar DRG were exposed. Two DRG (L4, L5) at each side and a specimen of the spinal cord were dissected. The sciatic nerve was exposed at each side by a slanting incision from the sciatic notch to the knee bend and a specimen of approximately 1 cm was dissected.

Freshly dissected tissue samples were immediately submerged into the embedding medium (Tissue-Tek O.C.T. Compound, Sakura Finetek Europe B.V., The Netherlands) and deep frozen in liquid nitrogen. Frozen tissue blocks were stored in sealed freezing vials at -80°C.

4.5 Frozen tissue sections

Frozen tissue blocks were placed into the chamber of the cryostat (2800 Frigocut, Reichert-Jung), allowed to equilibrate to the chamber temperature (-21°C) for about 30 minutes, bound with the embedding medium to the specimen block and mounted on the stub. The stub temperature was kept at –19°C while cutting. 7 µm thick tissue sections were immobilized onto aminosilan-coated microscope slides and dried at 25°C for 24 hours. The samples were stored at – 80°C until assessed in the ICA.

4.6 Haematoxylin-eosin (HE) staining of frozen tissue sections

Frozen tissue sections were warmed up to room temperature and fixed in an acetone bath at 25°C for 10 min. Subsequently the slides were immersed into the aqueous haematoxylin solution (2 g haematoxylin/l, 0.2 g $NaIO_3$/l, 17.6 g $KAl(SO_4)_2$/l) for 5 min at 25°C. Following that, the slides were rehydrated for 10 min and stained in 1% ethyl alcohol / eosin solution for 5 min. After a short water bath the slides were dehydrated in 70% ethyl alcohol, in 96% ethyl alcohol, in 100% ethyl alcohol and in xylene by immersing in each solution twice and finally embedded (Entellan, Merck). Morphological examination of the samples was performed by light microscopy (Axiovert 135, lens Plan-Neofluar 40x / 0.75, Zeiss).

4.7 Preparation of the monoclonal antibodies against Pt-DNA adducts

The monoclonal antibodies (MAB) against cisplatin-induced Pt-DNA adducts have been kindly provided by Dr. B. Liedert, Institute of Cell Biology, University of Essen. The generation and characterization of the MABs are described elsewhere (Liedert, 2001).

4.8 The Immuno-Cytological Assay (ICA) optimized for the single cell quantification of cisplatin-induced Pt-DNA-adducts in different cell types of the nervous system

4.8.1 Tissue immobilization

A special pretreatment of microscope slides keeps the tissue sections properly attached to the slide surface and prevents them from washing away during the ICA procedure. For this purpose the slides were coated with 2% 3-aminopropyl-triethoxysilan (aminosilan) solution in acetone at 25°C for 2 min, washed in acetone at 25°C and dried at 42°C for one hour.
Frozen tissue sections were placed onto the coated slides, dehydrated at 25°C for 24h and stored at – 80°C until assessment.

4.8.2 Immuno-histochemical identification of cell types

In order to perform cell type specific quantification of Pt-DNA adducts in nervous tissue, the neurons as well as glial or satellite cells were labeled immunohistochemically.
The frozen tissue sections of the DRG or spinal cord were fixed in methanol at – 20°C for 12h and subsequently rehydrated for 10 min in PBS, pH 7.4, at 25°C. To permeabilize the tissues and allow a good penetration of antibodies and staining agents the slides were treated in a 0.1% Triton X-100 / PBS at 25°C for 10 min, followed by washing in a PBS at 25°C. The non-specific protein binding was inhibited by incubation with 1% casein / PBS solution for 30 min at 25°C, followed by two washes in PBS. In the next step 100µl of Mouse IgG1 monoclonal anti-CNPase (marker for oligodendrocytes and satellite cells, 5µg/ml in 1% casein / PBS, Sigma) were applied to each slide and the samples were incubated for 90 min at 37°C in the humidified chamber. Subsequently the slides were washed in 0.05% Tween 20 / PBS for 2 min at 25°C and then in PBS for 5 min. In the following step 100µl of "ALEXA FLUOR 488" – Goat anti-Mouse IgG (H+L) (5µg/ml in 1% casein / PBS, Molecular Probes) were applied to each slide and the samples were incubated for 1 h at 37°C in the humidified chamber. Subsequently the slides were washed in 0.05% Tween 20 / PBS for 2 min at 25°C and in PBS for 5 min. This was followed by an incubation with the "NeuroTrace™ 530/615 red fluorescent Nissl Stain" (marker for neurons, Molecular Probes), 100µl per slide (1:50 dilution in PBS) for 20 min at 25°C in the humidified chamber. Subsequently the slides were washed with two changes of PBS for 5 min each, followed by a 2h-long wash in PBS at 25°C. The DNA-counterstaining was then performed with 1µg/ml DAPI in PBS for 30 min at 25°C. Consequently the slides were washed in PBS at 25°C. Finally, the slides were mounted in dithioeritritol solution (4,4mg/ml dithioeritritol (Sigma) in 60% of 50mM Tris/HCl, pH 8.2, 30% of glycerol, 10% of 10% polyvinyl alcohol) and covered with coverslips.
The digital images of the sections and the cell type identification were performed by means of laser-scan microscopy (Axiovert 100 with the LSM 510 digital measuring and evaluation system, Zeiss). The DRG and the spinal cord neurons were determined by red, glial or satellite cells – by green fluorescence, the cell nuclei – by DAPI / blue fluorescence.

The images were stored electronically. The coverslips were then gently removed and the sections were rehydrated and kept in PBS at 25°C for further assessment in the ICA.

4.8.3 Immuno-Cytological Assay

The tissue sections of the DRG, spinal cord or sciatic nerve were fixed in methanol at – 20°C for 12h and subsequently rehydrated for 10 min in PBS, pH 7.4, at 25°C. An RNA-digestion was performed with 100 µl of RNAse solution per slide (200 µg/ml RNAse A & 50 U/ml RNAse T1 in PBS) for 1 h at 37°C in the humidified chamber followed by one wash in PBS at 25°C. To provide a better penetration of antibodies in the tissue structures with high myelin content the probes of the spinal cord and the sciatic nerve first were treated in 0.1% Triton X-100 / PBS at 25°C for 10 min, followed by washing in PBS at 25°C. As a next step an alkaline permeabilisation of cytoplasmic and nuclear membranes was performed (60% 70 mM NaOH / 140 mM NaCl, 40% methanol) for 5 min at 0°C in container, followed by wash in PBS at 25°C. The DRG sections were undergoing an alkaline permeabilisation as described above immediately after the RNA-digesting step without pre-treatment with Triton X-100. In the following, a two-step proteolytic cleavage of cytoplasmic and nuclear proteins was performed. At first the sections were incubated with pre-warmed pepsin solution, 100 µl per slide (DRG: 300 µg/ml in 20mM HCl; the spinal cord: 600 µg/ml in 20mM HCl; the sciatic nerve: 1000 µg/ml in 20mM HCl) for 10 min at 37°C in the humidified chamber, followed by a wash in PBS at 25°C. In a second step the samples were treated with pre-warmed proteinase K solution, 100 µl per slide (100 µg/ml of proteinase K in 20mM Tris/HCl, 2mM $CaCl_2$, pH 7.5) for 10 min at 37°C in the humidified chamber. Consequently the slides were washed in 0.2% glycine / PBS for 10 min at 25°C. Non-specific protein binding was inhibited by incubation with 1% casein / PBS solution for 30 min at 25°C. Then the samples were incubated with anti-(Pt-DNA) MABs, 100 µl per slide (0,1 µg/ml in PBS, 1% casein, 200 µg of sonicated calf thymus DNA / ml) for 2 h at 37°C in the humidified chamber. Subsequently the slides were washed in 0.05% Tween 20 / PBS for 2 min at 25°C and then in PBS for 5 min. Then the incubation with FITC- Goat IgG $F(ab')_2$ anti-(Rat [IgG + IgM (H+L)]), 100 µl per slide, was performed (6.5 µg/ml in PBS, 1% casein, Dianova) for 45 min at 37°C in the humidified chamber, followed by washing of the slides in 0.05% Tween 20 / PBS for 2 min at 25°C and then in PBS for 5 min. To amplify the immunofluorescence signal a “sandwich” immunostaining with a cascade of secondary antibodies was performed. First, 100 µl of “ALEXA FLUOR 488” – Rabbit IgG anti-(FITC) were applied to each slide (5 µg/ml in PBS, 1% casein, Molecular Probes) and the probes were incubated for 45min at 37°C in the humidified chamber. Subsequently the slides were washed in 0.05% Tween 20 / PBS for 2 min at 25°C and in PBS for 5 min. This was followed by an incubation of the samples with “ALEXA FLUOR 488” – Goat anti-(Rabbit IgG (H+L)), 100 µl per slide (5 µg/ml in PBS, 1% casein, Molecular Probes) for 45min at 37°C in the humidified chamber. Subsequently the slides were washed in 0.05% Tween 20 / PBS for 2 min at 25°C and in PBS for 5 min. The DNA-counterstaining was then performed with 1µg/ml DAPI in PBS for 30 min at 25°C. Consequently the slides were washed in PBS at 25°C.

Finally, the probes were embedded with a densification compound “VECTASHIELD” (Vector). The quantification of immune- and DNA-fluorescence was performed by means of microscope-coupled digital image analysis system (fluorescent microscope Axioplan, lens Plan-Neofluar 40x / 0.75, Zeiss; mercury lamp HBO 100 W; DAPI-filter: excitation 365 nm, emission 397 nm; FITC / ALEXA 488- filter: excitation 450 – 490 nm, emission 515 – 565 nm; charge-coupled device camera C4880, Hamamatsu; ACAS 6.0 Cytometry Analysis System, Ahrens Electronics). Adduct levels in the nuclear DNA of individual cells were calculated by normalizing antibody-derived fluorescence signals to the corresponding DNA content of the same cell nucleus and expressed as relative units (RU). Data were assigned to specific cell types as determined by histochemistry

4.9 Electrophysiological examination of motor and sensory nerve fibers

All electrophysiological examinations were carried out under general anesthesia (Inactin®, Sigma, 50 mg/kg body weight, i.p.). To minimize effects of body temperature differences on conduction velocity, animals were placed over a warm flat steamer controlled by a hot water circulating pump, and the hind paw skin temperature was maintained at 37-38°C. The tibial and sciatic nerves were stimulated percutaneously through a pair of monopolar needle electrodes placed at the ankle and sciatic notch, respectively. Square-wave electrical pulses of 0.7-msec duration were delivered by Pulsemaster™ A 300 stimulator through an A 365 stimulus isolation unit (World Precision Instruments, USA). The stimuli were applied starting from 0.1mA up to 25% above the amperage that gave a maximal response.

Upon stimulation of the mixed sciatic or tibial nerve, two responses occur. The M-response is a direct motor response with short latency due to stimulation of the á-motor fibers. The H-reflex is an indirect response with long latency due to stimulation of the afferent proprioceptive Ia fibers. The Ia fibers monosynaptically excite á-motor neurons in the spinal cord (figure 18). At lower stimulation intensities only the thicker afferent fibers are excitated and only an H-reflex is elicited. At increasing stimulation intensities both H- and M-responses occur, while at repeated supramaximal stimulation the H-reflex is absent due to antidromic conduction in the motor axons (De Koning et al., 1989).

The compound muscle action potentials (CMAPs) elicited by orthodromic conduction (M-response) and by the monosynaptic reflex arc (H-response), were recorded from the third interosseous muscle of the hindfoot with a pair of microneedle recording electrodes. Mice were grounded by means of a needle electrode inserted between the stimulation and recording points. After amplification (INH 4-channel differential amplifier, Science Products GMBH) the evoked action potentials were sampled and analyzed by means of a CED micro1401 system with the “Signal for Windows Version 2.05” software (Cambridge Electronic Design Limited, UK), running under Windows 98 on an IBM PC.

The amplitude of M- and H-responses was measured from baseline to the maximal negative peak and expressed in mV. The response latencies (ms) were defined as the interval between the stimulus artifact and the beginning of the M- or H-response. The distance between the two stimulation points was measured over the skin with the hip and knee in a flexed position. Motor (MNCV) and H-reflex-related sensory nerve conduction velocity (SNCV) were calculated as follows:

MNCV [m/s]=distance (sciatic notch-ankle) / latency (M-response sciatic notch – M-response ankle);

SNCV [m/s]=distance (sciatic notch-ankle) / latency (H-response ankle – H-response sciatic notch).

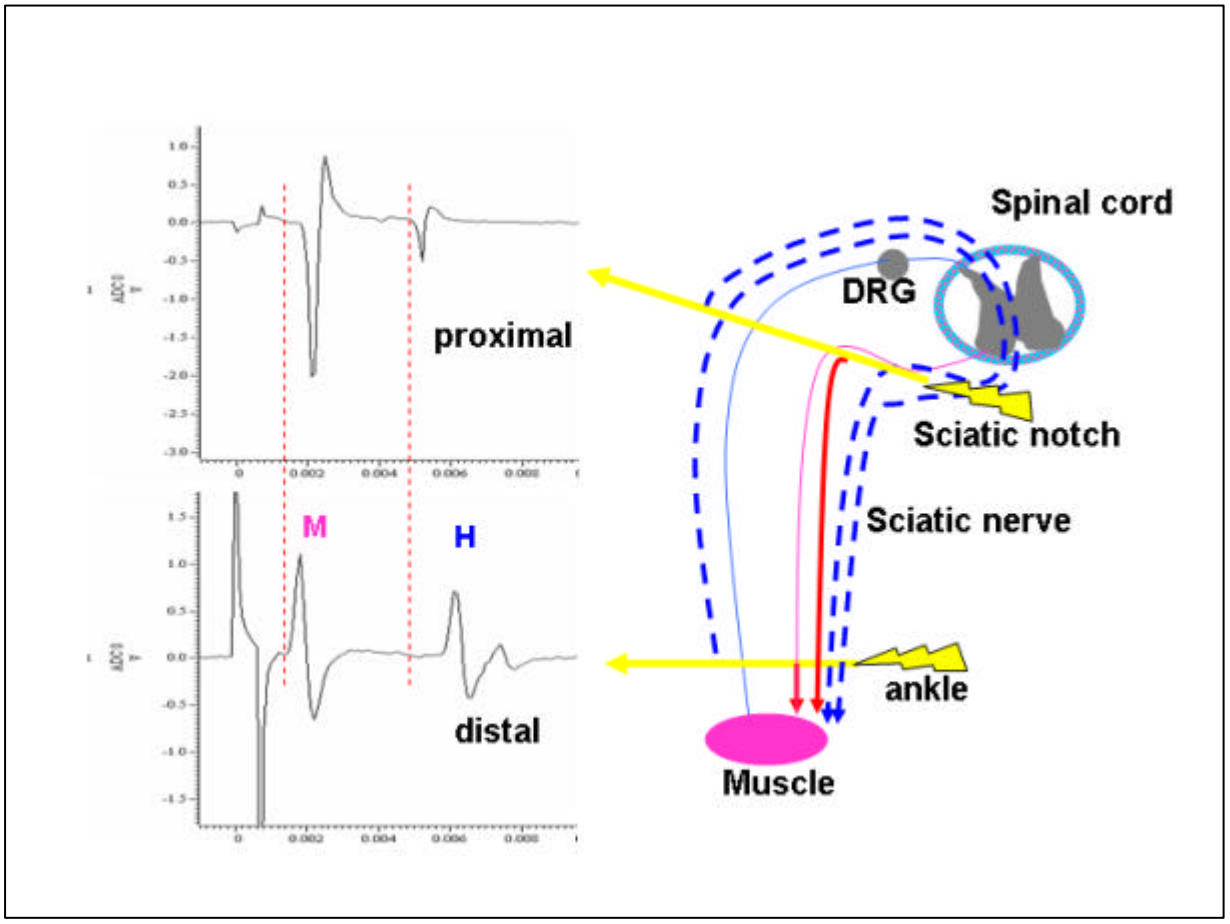


Figure 18 Electrophysiological examination of motor and sensory nerve fibers: schematic illustration of the test model. The pictures show the M- and H-responses recorded from small muscles of the foot by a pair of microneedle electrodes after stimulating at the sciatic notch (proximal) and ankle (distal), respectively.

4.10 Data analysis

In all experiments for quantitative analysis of cisplatin-induced DNA lesions in different cell types of central and peripheral nervous system two treated mice and one control were used per time point. From each mouse four dorsal root ganglia (lumbar), the lumbar part of the spinal cord and both sciatic nerves were dissected. From each DRG 30 sections, from the spinal cord 100 sections and from the sciatic nerve 50 sections were prepared. 10 sections of each tissue sample were analyzed chosen at random. In total a minimum of 200 cells of each cell type per animal were analyzed. The calculation of Pt-DNA adducts levels was made using “SigmaPlot 4.0” software (Jandel Scientific). The results are given in relative units (mean +/- SE).

In all electrophysiological experiments ten treated mice and ten age-matched controls were used per time point.

4.11 Statistics

Statistical significance of the relationships between experimental data was assessed by means of ANOVA for repetitive measurements and by the multiple *t*-Test post-hoc analysis performed using SPSS 9.1 software (SPSS Inc.). A two-sided *P* value of < 0.05 was regarded as indicating statistical significance.

SUMMARY

Neurotoxicity with polyneuropathy as its predominant clinical sign is the major dose limiting side effect of chemotherapy with DNA reactive drugs such as cisplatin. The underlying mechanisms, however, are still not clear. Platinum compounds are known to exert their antineoplastic activity by forming distinct Pt-DNA adducts. Both the DNA repair rates and the extent of tolerance to persisting lesions determine the cell type specific sensitivity to cisplatin. In this report, DNA damage and repair are investigated with regard to their contribution to cisplatin-induced neurotoxicity in a mouse model. As the nucleotide excision repair (NER) pathway is considered to be an important mechanism for the processing of Pt-DNA lesions, its role was studied in an experimental setting with NER-deficient mice lacking functional XPA or XPC proteins. Employing monoclonal antibodies, structurally defined DNA lesions were quantified in various cells of the central and peripheral nervous system in wild type (WT) and repair deficient mice at different time points after a single exposure to cisplatin. In order to clarify, if accumulation of DNA lesions correlates with functional impairment, adduct quantification and electrophysiological studies were assessed in WT and $XPA^{-/-}$ mice under chronic cisplatin administration. The accumulation of Pt-DNA-adducts was dose dependent and two-fold higher in dorsal root ganglion (DRG) neurons and satellite cells (outside the blood-brain barrier) than in the same cell types of the spinal cord. Furthermore satellite and glial cells showed a higher adduct burden than corresponding neurons. Pt-DNA lesions peaked in all cell types of nervous tissue 24 hours after treatment. In the cells of DNA repair proficient WT mice adducts were removed by DNA repair within 7 days to a level, which represented 30% of the initial burden. Both NER deficient strains showed increased Pt-DNA adduct accumulation due to the insufficient repair of early monovalent adducts, which are known to occur as intermediate structures during cisplatin-DNA interaction. As a consequence, a massive loss of DRG cells was detected 48h after cisplatin application. While in the absence of active XPA protein some reduction of DNA lesions still could be observed, the loss of XPC function resulted in a complete lack of repair and in long term persistence of high adduct levels. Under chronic treatment, $XPA^{-/-}$ mice showed a significant increase in persisting DNA lesions compared to the WT counter points due to an impaired adduct removal in these cells. Furthermore, the enhanced persistence of Pt-DNA adducts in DRG cells of DNA repair deficient mice was strongly associated with an early functional impairment of sensory nerves: both, the amplitude of H-reflex and sensory nerve conduction velocity decreased significantly, whereas NER competent WT mice, bearing 35% less platinum-DNA lesions in their DRG cells, remained electrophysiologically intact under the same cumulative doses. Thus, there was a strong correlation of Pt-DNA adduct levels in DRG neurons with the extent of their morphological damage and with electrophysiological changes in sensory nerve fibers. The obtained data suggest that accumulation of DNA adducts is the crucial factor in the development of cisplatin-induced sensory polyneuropathy. Elucidation of these pharmacokinetic and pharmacodynamic processes is the basis for the development of neuroprotective substances which should prevent patients from the therapy-induced neuronal damage without blocking anti-neoplastic effects of cisplatin in malignant cells.

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APPENDICIES

Devices

Centrifuge:	Biofuge B‘‘ (Heraeus Christ)
Cryostat	“2800 Frigocut“ (Reichert-Jung);
Electrical pulse stimulator	“Pulsemaster A 300” (World Precision Instruments) Electrical stimulus isolation unit “A 365” (World Precision Instruments)
Electrical signal amplifier	“INH 4-channel differential amplifier” (Science Products)
Electrical signal sampling system	“CED micro1401” (Cambridge Electronic Design) Software “Signal for Windows 2.05” (Cambridge Electronic Design)
Fluorescent microscope	“Axioplan” (Zeiss) Lens “Plan-Neofluar“ 40x / 0.75, (Zeiss) Mercury lamp “HBO 100 W“ (Zeiss) CCD camera “C4880“ (Hamamatsu) “ACAS 6.0“ Cytometry Analysis System (Ahrens Elektronics)
Fluorescence plate reader	“FL 500“ (Bio-Tek Instruments) PC-program “FL 500 1.D.1“ (Bio-Tek Instruments)
Heater	“PMC digital dry block heater” (Roth)
Laser-scan microscope	“Axiovert 100“ (Zeiss) Digital measuring & evaluation system “LSM 510“ (Zeiss)
Light microscope	“Axiovert 135” (Zeiss) Lens “Plan-Neofluar” 40x / 0.75 (Zeiss)
Microwave	(Bosch)
PCR-device	“MJ Research PTC 200“ (Biozym)
Power Supply:	“Electrophoresis power supply‘‘ (Renner GmbH)
Spectrophotometer	“Model 150-20“ (Hitachi);
Stereomicroscope	“Stemi SV 6” (Zeiss) Lens “W-Pl” 10x / 1 (Zeiss)
UV-transilluminator	(Bachmann)

PC programs

Control programs see Devices
Excel 2002 (Microsoft)
iPhoto Plus 4 (Ulead Systems Inc.)
PowerPoint 2002 (Microsoft)
Sigma Plot 4.0 (Jandel Scientific)
SPSS 9.1 (SPSS Inc.)
Word 2002 (Microsoft).

Chemicals

Chemicals were at least “p.a.” grade and obtained from Merck, with the following exceptions:
Agarose (Biozym)
Aminopropyl-triethoxysilan (Sigma)
Block protein “Rad-Free“ (Schleicher & Schuell)
Cisplatin “Platinex“ (Bristol Arzneimittel GmbH)
dATP, dCTP, dGTP, dTTP-mixed for PCR (Amersham Pharmacia Biotech)
Dithioeritritol (Sigma)
Ethidiumbromide (Serva)
Calf thymus DNA (Roche)
“NeuroTrace™ 530/615 red fluorescent Nissl Stain” (Molecular Probes)
Tissue-Tek O.C.T. Compound (Sakura Finetek Europe B.V.)
Triton X-100 (Sigma)
Tween 20 (Sigma)
Embedding medium “Vectashield“ (Vector)

Buffers and solutions

The buffers and solution used in the experiments were composed as described in Materials and Methods (4.1), except following compounds:

PBS	10mM Na_2HPO_4
	0.13M NaCl
	pH 7.4
TAE-buffer	0.04M Tris-acetate
	0.001M EDTA; pH 7.5
TE-buffer	10mM Tris
	1mM EDTA; pH 7.5

Kits

DNA-isolation	“DNeasy Kit“ (Qiagen)
DNA-quantification	“SYBR Green I“ (Molecular Probes)

Molecular weight standards

DNA size-standards „GeneRuler 50 bp, 100 bp and 1 kb DNA Ladder“ (MBI)

Enzymes

AccuTherm-Polymerase (GeneCraft)
Pepsin (Roche)
Proteinase K (Merck)
RNAse A (Roche)
RNAse T1 (Roche)

Antibodies

“ALEXA FLUOR 488” – Goat anti-Mouse IgG (H+L) (Molecular Probes)
“ALEXA FLUOR 488” – Goat anti-(Rabbit IgG (H+L)) (Molecular Probes)
“ALEXA FLUOR 488” – Rabbit IgG anti-(FITC) (Molecular Probes)
FITC- Goat IgG F(ab´)$_2$ anti-(Rat [IgG + IgM (H+L)]) (Dianova)
Mouse anti-CNPase IgG1 MAB (Sigma)

PCR primers

MWG Biotech:
Primer A (Upper primer: wild type)
5´ GTG GGT GCT GGG CTG TCT AA 3´
Primer B (Lower primer: wild type / knockout)
5´ATG GCG TGG GTT CTT CTT C 3´
Primer C (Upper primer: knockout):
5´ ATG GCC GCT TTT CTG GAT TC 3´

Abbreviations

A	adenine
AAS	atom absorption spectrometry
ABC	ATP binding cassette
ATP	adenosine triphosphate
BAX	Bcl2-associated X protein
bp	base pair
BBB	blood-brain barrier
C	cytosine
CMAP	compound muscle action potential
cMOAT	canalicular multispecific organic anion transporter
CNS	central nervous system
DNA	desoxyribosenucleic acid
dNTP	desoxyribosenucleotide triphosphate
DRG	dorsal root ganglion
EDTA	ethylendiaminetetraacetate
ELISA	enzyme-linked immuno-sorbent assay
EMG	electromyography
G	guanine
HE	Haematoxylin-eosin
ICA	Immuno-Cytological Assay
ICP-MS	inductively coupled plasma mass spectroscopy
i.p.	intraperitoneal
kb	kilobase pair
k.o.	knockout
LD_{50}	50% of the lethal dose
MAB	monoclonal antibody
MNCV	motor nerve conduction velocity
MRP	multidrug resistance protein
NER	nucleotide excision repair
NGF	nerve growth factor
NMR	nuclear magnetic resonance
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PNP	peripheral polyneuropathy
Pt	platinum
RNA	ribonucleic acid
RU	relative unit
SC	spinal cord
SN	sciatic nerve
SNCV	sensory nerve conduction velocity
TAE	Tris-acetate-EDTA
T	thymine
TE	Tris-EDTA
Tris	Tris-(hydroxymethyl)-aminomethane
U	relative enzyme unit
UV	ultraviolet
WT	wild type
XP	Xeroderma pigmentosum

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